Ecophysiological study of *Centella asiatica* (Linn.) Urban of Nepal

By

Anjana Devkota

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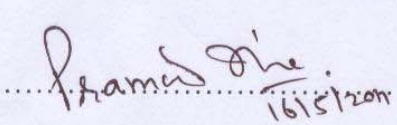
CERTIFICATE

We have the pleasure of forwarding the dissertation entitled "Ecophysiological study of *Centella asiatica* (Linn.) Urban of Nepal" by Ms. Anjana Devkota, Lecturer, Central Department of Botany, Tribhuvan University, Kathmandu for the fulfillment of the degree of doctor of Phillosophy in Botany. The dissertation is based on original research work carried out under our supervision and has not been submitted to a degree to any other university.



Research Supervisor

Pramod Kumar Jha, Ph.D.

Professor, Central Department of Botany

Tribhuvan University, Kathmandu, Nepal

Research Co-Supervisor

Gabriella Innocenti, Ph.D

Professor, Department of Pharmaceutical Sciences, University of Padova, Padova, Italy.

Date: December 28, 2010

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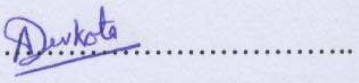
I hereby declare that the work presented in this dissertation has been carried out and put together by myself. I have not submitted any part of this work for any form of degree. All information cited in this work has been specifically acknowledged and credited to the respective authors or institutions as references.



Anjana Devkota

Central Department of Botany

Tribhuvan University, Kathmandu

This work has been dedicated to my parent

Babu Ram Devkota and Netra Kumari Devkota

and

my husband Chandra Kanta Bhandari

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ACRONYMS

%:	Percentage
°C:	degree Celsius
a.u:	Arbitrary units
Asl:	Above Sea level
C:	Central
CDB:	Central Department of Botany
cm:	centimeter
CTAB:	Hexadecyl-trimethylammonium bromide
d.w:	Dry weight
DAD:	Diode Array Detector
DNA:	Deoxy ribonucleic acid
DPR:	Department of Plant Resources
E:	Eastern
ELSD:	Evaporative Light Scattering Detector
FYM:	Farmyard Manure
g:	gram
$GA_{3:}$	Gibberellic acid
GC-FID:	Gas Chromatography- Flame Ionisation Detector
GC-MS:	Gas Chromatography -Mass Spectrometry
h:	Hours
HPLC:	High Performance liquid Chromatography
m:	meter
MAPs:	Medicinal and Aromatic Plants
MOFSC:	Ministry of Forests and Soil Conservation
MPs:	Medicinal Plants
N:	Nitrogen
OC:	Organic Carbon
OM:	Organic matter
PCR:	Polymerase Chain reaction
ppm:	Parts per million
RAPD:	Random amplified polymorphic DNA
RT:	Room temperature
SLA:	Specific Leaf Area (cm^2/g)
TCM:	Traditional Chinese Medicine
TTC:	2,3,5-triphenyltetrazolium chloride
TU:	Tribhuvan University
UPGMA:	Unweighted pair group method with arithmetic averages
UV light:	Ultraviolet light
W:	Western
WHO:	World Health Organization

ABSTRACT

Centella asiatica (L.) Urban, commonly known as Indian Pennywort, is an important ethnomedicinal plant of tropical to subtropical region. It is a clonal perennial herb with a wide range of enthomedicinal uses such as blood purifier, memory enhancer, anticancer, antidepressive etc. Distribution pattern, abundance, life history traits, leaf nitrogen content, genetic diversity of population, and quantification of eight secondary metabolites of 21 populations of *Centella asiatica* from different habitats and regions of Nepal were studied. Effects of different environmental factors (moisture, soil texture, light and shading) and integrated manuring on growth traits and yield of *C. asiatica* were determined in pot-grown plants treated under randomized block design.

Centella asiatica grows in a wide range of habitats from shady grassland, open grassland, to open agricultural land in tropical to temperate area in Nepal. In terms of density and plant biomass, partially shaded grassland was the most suitable natural habitat for *C. asiatica*. Density and biomass yields varied significantly with habitat types, with mean value of 72.53 pl/m^2 and 37.95 g/m^2, respectively. The flowering peak was recorded in April-June and it little varies in different habitats. The freshly collected seeds had the highest viability which declined progressively as the duration of storage increased. Pretreatment like soaking seeds with GA_3 prior to sowing reduced the time required for initiation of germination. Seed germination of *C. asiatica* was affected adversely by salinity. Aqueous extract of some invasive plants viz. *Chromolaena odorata, Parthenium hysterophorus, Ageratum conyzoides* and *Xanthium strumarium* had inhibitory effects on germination, which threaten the population density of *C. asiatica* in nature.

The pot experiment with different moisture levels (125% FWC (surplus water), 100% FWC, 70% FWC and 30% FWC) showed that the dry matter production and yield in *Centella asiatica* was highest in 100% FWC, followed by 70% due to higher growth characteristics such as number of primary branches, leaves, and leaf chlorophyll contents. Growth under four levels of shade (0, 30, 50 and 70%) showed that dry matter production and yield was significantly higher in 30% shade, followed by 50 %. Asiatic acid was significantly higher in 70% shading. There was no significant effect of shading on other measured secondary metabolites. Growth traits and yield of *C.*

asiatica was significantly higher in sandy loam soil than clay-loam and pure sand. All measured secondary metabolites were significantly higher in sand than in clay-loam and sandy loam. The pot experiment with different integrated manuring conditions (Urea: FYM, 100:0; 75:25; 50:50; 25:75; 0:100; and control - no manure) showed higher yield and better growth traits of *Centella asiatica* in integrated manuring .

Concentration of all secondary metabolites measured in present study was significantly higher in open agricultural land than in shady and open grasslands. Mean concentration of asiaticoside, the most important bioactive component of *C. asiatica*, was 1.8% (dw). Concentration of secondary metabolites was higher in samples from central Nepal than western and eastern Nepal. Asiaticoside content was inversely related with the altitude of samples collected. Asiaticoside content was higher in samples collected from 150-600 m asl. Thus, a negative correlation was observed between altitude and asiaticoside content while the opposite was for quercetin-3-O- glucuronide content. Concentration of secondary metabolites was higher in wild than in transplanted samples. Essential oil yield of *Centella asiatica* from different habitats ranged from 0.10 (open grassland) to 0.12% (shady grassland). Total yield of essential oil was higher in samples from partially shaded habitat, but concentration of major components was higher in open agricultural land.

Genetic diversity study of 21 different populations of *Centella asiatica* was carried out by morphological and molecular marker (RAPD). Morphological characters were significantly different among 21 populations. Two distinct morpho-types of *Centella asiatica* were clearly distinguished in dendrogram based on morphology; one with small leaves, dentate to serrate margin, and creeping form, and the other with large leaves, crenate to entire margin, and erect form. Molecular marker data showed similarity coefficient from 0.52-0.91 among the population indicating a moderate diversity of *C. asiatica* in Nepal.

In conclusion, morphology, genetic character and active phytochemicals in *Centella asiatica* at different habitats and ecological regions of Nepal varied. Transplanted samples had lower amount of secondary metabolites than in wild samples.

Key Words: Distribution, life history traits, medicinal plant, environmental factors, genetic diversity, morphological and molecular markers (RAPD).

CONTENTS

1. INTRODUCTION

1.1 Background

Plant based remedies have always been an integral part of traditional medicine worldwide. The widespread use of herbal remedies and healthcare preparations, as described in ancient texts including the Vedas, holy Koran and the Bible, are obtained from commonly used traditional medicinal plants. A rich heritage of knowledge on preventive and curative medicines was even available in ancient scholastic work included in the Atharva Veda, Charaka Sushruta, etc. Medicinal plants are essential natural resource which constitutes one of the potential sources of new products and bioactive compounds for drug development (Gangwar *et al.* 2010). Globally the number of plants with known medicinal values has been estimated to be about 70,000 (Schippmann *et al*. 2006) and its number has been increasing steadily. The World Health Organization has estimated that more than 80% of the world's population in developing countries depends on herbal medicine for primary healthcare needs (Vines 2004). The usage of herbal medicine is increasing at a rate of 10 to 20% annually (Pick Kiong 2004). The increase in the demand for herbal medicines in recent years is probably due to insignificant side effects compared to synthetic drugs and antibiotics (Banerjee *et al.* 1999). Using traditional ethnomedicinal knowledge as starting point, scientists were able to isolate bioactive natural products from plants to be used successfully as drugs (Cordell 2000, Ji *et al*. 2009) saving lives of millions of people every year (Roberson 2008).

The therapeutical property of medicinal plants depends on physiologically active chemical compounds produced in the plant as secondary metabolites like glycosides, terpenes, alkaloids, phenolics, steroids, coumarins, saponins, etc (FAO 1993, Walton and Brown 1999). Hence these plants are generally referred to as `natural bio-chemical factories' or `chemical goldmines'. Secondary metabolites are produced during the growth and development of plants along with the adaptation to outer environment, as a rule they are the main active ingredients in medicinal plants and ensure the quality of crude drugs. Since biogenesis is quite complex, the production and accumulation of secondary metabolites are influenced by various biotic and abiotic factors either from gene or environments, the complexity may affect quality control of crude drugs and

utilization of the active ingredients. A number of medicinal principles have already been identified and no synthetic substitutes are currently available for many of them (Wijesekera 1991, Kumar *et al.* 1997).

Due to their commercial prospects and high chances of finding new drugs against serious diseases, pharmaceutical companies and research institutions, respectively, have developed renewed interest in biomedical researches of medicinal plants. This has been combined with increasing popularity of herbal based products in western society, leading to steady increase in volume of medicinal plants being collected from wild for trade (Koul and Wahab 2004). Therefore, additional ethnomedicinal plants of Himalayas may enter into trade in the future.

During the past decade, a dramatic increase in export of medicinal plants attests worldwide interest in natural products made from many of these medicinal and aromatic plants. This demand has while on one hand managed to give an alternative source of income to many of the rural poor, on the other hand it has also managed to exacerbate the already existing problem of over-exploitation of medicinal plants from the wild. Open access to medicinal plants in the wild is perhaps one of the main reasons for the current unsustainable levels of harvesting. Market driven extraction of medicinal plants from wild without considering their life history and the amount that can be extracted sustainably has threatened some medicinal plants of the Himalayas to the level of local extinction. The combination of slow growth of many medicinal plants with unsustainable, destructive harvesting practices of uprooting whole plant for a single economically important part has led to the rapid decline of this population in the wild. While some have been threatened due to unsustainable harvest from wild population for trade (Ghimire *et al.* 1999, 2005; Kala 2000, 2005), others are naturally rare and populations have been declining even if they have been used only for traditional health care (Kala 2005, Shrestha and Jha 2010). One fifth of the plants with known medicinal uses has been estimated to be threatened (Schippmann *et al.* 2006), but most of the information related to the status of these medicinal plants have been based on experts' perception rather than empirical data (Dhar *et al.* 2000, Mulliken and Crofton 2008).

Protection of wild population and cultivation for commercial purpose are two important strategies, which can prevent the species from being extinct. Cultivation is the best

option to decrease harvest pressure on wild population (Canter *et al.* 2005). Cultivation and research permits selection of better species and information about their respective properties, improved quality, effective conservation and increased prospects for genetic improvement. However, there are some major problems in cultivation such as decline in medicinal value of plant materials from cultivation (Hamilton 2003, Schippmann *et al* 2002). A successful cultivation without any decay in medicinal value of the plant may need replication of wild habitat condition in the farm land, which is virtually impossible but can be maximized if we have detail information on habitat requirements and plant growth performance at different habitat in wild condition. Biological study is a prerequisite to develop management plan for medicinal plants (Schippmann *et al*. 2002). We have to know the growth requirements of plants like optimum condition of light, soil texture, soil moisture condition as well as nutrient requirement. However, except for a few high-profile taxa, population details are largely lacking for most Himalayan medicinal plants (Dhar *et al*. 2000), and life history strategies have been studied only for a few species of Nepal Himalaya (Ghimire *et al.* 2005, Shrestha and Jha 2010).

Ecophysiological study of plants can help to understand factors that govern growth rate, reproduction, survival, abundance and geographical distribution of plants. Such study can also help to understand the functional significance of specific plant traits. Plant growth rate, allocation of growth among organs, photosynthesis, and nutrient use efficiency represent quantitative traits that are important for plant survival in natural environments. Therefore, plant ecophysiologists commonly measure these traits as indicators of plant response to the environment (Bazzaz 1996). Ecophysiological studies in populational approach consider the differences between individuals, which is condition for the operation of natural selection (Bazzaz 1996). In the same way, Lüttge and Scarano (2004) also emphasized the importance of taking into account the intra-specific variability in ecophysiological studies, including phenotypic plasticity and its importance on the development of new species. Phenotypic plasticity, usually defined as the property of genotypes of exhibiting different phenotypes in different environments, could delay speciation due to the protection of genotypes from a specific environmental pressure. It also could allow diversification in species that present an extensive ecological range resulting in specialized genotypes in particular habitats, the ecotypes. Phenotypic plasticity can be adaptive, improving the plant survival and

reproduction, or not, due to biochemical, physiological and developmental constraints (Pigliucci *et al.* 2006). The variability of ecophysiological traits among populations in some way can be a result of selection in response to environmental pressure and/or can be due to random factors, i.e. genetic drift. The analysis of both, genetic and phenotypic variation within populations can lead to better understanding of adaptation in direction to the occupying of different habitats. It is also important in plant ecophysiology to evaluate genetic regulation of these traits in order to understand the mechanisms by which plants have adapted to diverse environments. Ecophysiological information of medicinal plants in wild is essential to develop suitable agro-technology so as to minimize the decline in therapeutic quality due to cultivation.

Centella asiatica, an important tropical medicinal plant, is widely distributed in Nepal from eastern to western upto 2200 m above sea level (masl). In the present work, ecophysiology of *Centella asiatica* was studied in their natural habitat range in Nepal. The plant is widely distributed in tropical and sub tropical region of the world (Press *et al.* 2000). *C. asiatica* is commonly occur species to south-southeast Asia, Australia, Madagascar and Southern and Central Africa (Tiwari *et al.* 2000b) and has been used in traditional medicine as memory enhancer, blood purifier, tonic, stomachic and against jaundice, bronchitis, dysentery and kidney stone (reviewed by Devkota and Jha 2008).Secondary metabolites present in this plant are known to have wound healing, vasodilatory, ulcer preventive and anticanceric activities (Cook and Samman 1996, Kan 1986, Shukla *et al.* 1999). Though the plant is widely distributed and extensively used as traditional medicine, availability and life history strategies of this taxon have not been studied in Nepal. Distribution, life history, plant environment interaction, leaf nitrogen content, soil nutrient status (nitrogen and organic carbon), factors effecting growth of plant (light, soil texture, water and manuring condition) and intraspecific variation (genetic diversity) among and within population of *C. asiatica* were studied with quantification of eight major bioactive phytochemical components (secondary metabolites).

1.2 Rationale and Implications

Mountains harbor a large number of medicinal plants, which have been used traditionally by local people for treatment of various diseases. These plants can be important source of natural products. Isolation and identification of active natural products from traditionally used medicinal plants help to add monetary value to these resources. Most of the medicinal plants have been harvested from the wild. In addition, over harvesting of selected medicinal plants that has been taking place in Nepal for trade, with no proper care for conservation has contributed to the depletion of many plants in the wild (Chaudhary 2000).

Ecophysiological information of medicinal plants in wild is essential for their cultivation, particularly for selection of soil type and microclimate. Information on genetic diversity is of great interest and importance to population geneticists and plant breeders (Badr *et al.* 2002). The study of genetic characteristics of plants may be used to identify suitable parents and to prevent progressive erosion in the genetic bases of breeding populations (Kölliker *et al*. 1999). In order to conserve genetic resources for plant improvement, it is necessary to preserve, maintain and document genetic diversity (Lane *et al.* 2000). Better understanding of genetic variation at the intraspecific level can help in identifying superior genotype(s) for crop improvement as well as evolve strategies for effective *in situ* and *ex situ* conservation programmes. Such empirical determination of genetic diversity can be obtained by evaluating morphological, physiological and biochemical traits.

Centella asiatica has been valued for its cooling effect and as a memory enhancer, blood purifier and uric acid reducer in traditional medicine of Nepal. Though this plant has a high potential for therapeutic purposes and trade, it has not got adequate attention from policy makers, researchers and traders in Nepal. Since the quantity and quality of medicinally important secondary metabolites in plants depend on habitat condition, it is essential to identify the particular set of environmental condition under which their accumulation is the highest. In this regard the study of distribution, life history traits and quantification of active phytochemicals of *Centella asiatica* from different environmental conditions was carried out. Detailed biological and phytochemical information of plant were not available. Hence, this study was conducted to generate information that would be valuable for sustainable harvesting and cultivation of the

plant. The assessment of genetic variability in natural populations can provide new insights into the evolutionary history and phylogenetic relationships of *C. asiatica*, and can address potential problems in cultivation practices (Vaughan 1989).

1.3 Hypothesis

Present work has been based on following general hypothesis.

i . *Centella asiatica* has intraspecific variation.

ii. Environmental condition governs the phytochemical constitutents in *C. asiatica* of Nepal.

1.4 Objectives

The specific objectives were:

i. To investigate the distribution of *Centella asiatica* in Nepal.

ii. To investigate intra-specific (population) variation in morphological characters and genetic pattern of *C. asiatica.*

iii. To analyse growth pattern of *C. asiatica* in different environmental conditions.

iv. To study the quantitative variation of bioactive phytochemicals (secondary metabolites) in *C. asiatica* growing under different environmental conditions.

1.5 Limitations

The major limitations of the present work were

- Limited resources and time did not allow covering entire vertical range of distribution in Nepal for sampling.
- The sample size for genetic study was relatively small due to resource limitation.

2. REVIEW OF LITERATURE

2.1 Medicinal Plants in Nepal

Nepal is a veritable treasure trove of medicinal plants. In spite of being a small country, the diversity of medicinal plants is high. The estimates of total number of medicinal plant species (MPs) found in Nepal differ widely according to authors and time. Department of Plant Resources (1970, 1984) compiled 571 species of MPs from Nepal. Shrestha *et al.* (2000) compiled database of 1624 species of MPs of Nepal. Baral and Kurmi (2006) compiled 1,792 species of MPs (including lichens and fungi). Recently Ghimire (2008) recorded 1950 species of MPs in Nepal, out of which 1906 species are vascular plants. Nepalese medicinal plants especially unique Himalayan herbs were famous even in ancient times. Among other goods traded during the lucrative trans-Himalayan trade between Nepal and Tibet, medicinal plants formed a large part. Many important traditional practices of eastern medicine including Ayurveda, Unani, Siddha, Chinese and Tibetan medicine (*Sowa Rigpa*) which has their basis in medicinal plants are practiced in Nepal. Apart from a small fraction consumed by the domestic and local market, the bulk of Nepalese MPs are exported to India, followed by China and abroad where they are in much demand probably due to their high bioactivity and medicinal efficacy. Apart from medicinal value, many of these plants also carry economic, cultural, aesthetic and religious significance in Nepal.

Medicinal plants of Nepal were widely traded across the boarders to Tibet as early as 600 AD (Sung and Yiming 1998). Presently, over 90 % of the total export from Nepal is to India and mostly in the crude form. Conservative estimates of the annual Nepalese alpine and subalpine medicinal plants vary from 480 to 2500 tons, with a total harvest value of US$ 0.8- 3.3 million (Olsen and Larsen 2003).

2.2 Traditional Medicine in Nepal

Nepal is a multiethnic and multilingual country, with more than 28 million people comprising of 59 different ethnic groups. Religions such as Hinduism, Buddhism, Bon and others are followed, and the people have strong belief on traditional herbal medicinal practice for health treatment, some of which is also related to religion. Throughout the long history, Nepalese people have used plants as a mainstay of everyday life. The history of the use of medicinal plants in the Himalayas is found in

the *Rigveda*. This work, written between 4500 and 1600 BC, is supposed to be the oldest repository of human knowledge and describes 67 plants (Malla and Shakya 1984). Thus for thousands of years, human being has been using plant products as a cure for various ailments. After the *Rigveda, Ayurveda* describes the medicinal importance of 1200 plants. The *Charak Samhita* (900 BC) and *Susruta Samhita* (500 BC) enumerate the art of surgery, therapeutics and medicines in details on the basis of *Atharveda*. Numerous literatures written in Nepali, Newari, and Sanskrit languages contain records of Nepali medicinal plants. The original "*Saushrut Nighantu*" written on palm leaves in Newari script and Sanskrit verses during Mandeva Era (879 AD), is said to be the oldest of these books. However, the knowledge of using these systems was accessed by Nepali *Vaidhyas* as early as about 879 AD (IUCN 2004). In addition to Ayurvedic system, medicinal plants are also codified in other traditional medical systems, including Chinese, Unani, Siddha, Homeopathy, etc (Sivarajan and Balachandran 1994, Lama *et al.* 2001).

Starting from hand-written pharmacopeia to modern research, information about medicinal plants of Nepal is widely scattered in a large number of publications. The Department of Ayurveda, Ministry of Health, HMG/Nepal in 1998 listed essential Ayurvedic drugs comprising of 339 preparations under 44 main heading of symptomatic diseases.

South Asia is home to many traditional systems of medicine. Ayurvedic methods date back to 5000 BC. Along with the Unani, Siddha and Tibetan systems, they remain an important source of everyday healthcare and livelihood for tens of millions of people. Medicinal and Aromatic Plants (MAP's), including trees, shrubs, grasses and vines, are a central resource for these traditional health systems, as well as for pharmaceutical (or allopathic) medicines. MAP's are widely used in Nepal as medicine, additives, beverages, cosmetics, sweeteners, bitters, spices, dying agents and insecticides. Crude-drugs are commonly given in the form of powder, decoctions, and infusions or in ointments. The powder is prepared from dried parts while infusions are extracted by boiling the plants in water. The dried plants are also prepared to smoke like cigarettes for the treatment of cough, cold and headache. The herbal medicines are applied externally on cuts, wounds, boils, pimples, ringworms, muscular swelling and dislocation of bones. Plants are also used as a hot baths for skin diseases. The single plant or plant parts such as root, rhizome, stem, leaf, bark, wood, gum, latex, ash,

flower, fruit and seed, or as mixture of different species of plants are recommended for treatment. The rural communities of Nepal have a long tradition of using plant resources for their various basic needs such as food, medicine, firewood, timber, fodder and agricultural tools. They collect plants from various habitats, such as forest, scrub, grassland and cultivated fields, and use them as crude drugs. Through their experience to diagnose and treat diseases they gained knowledge on the useful and harmful properties of these plants. Such knowledge forms a basis for a better and fuller utilization of the plant wealth. Since the population of Nepal has different ethnic groups, there are disparities and commonalities in the way of employing the same plant species and preparing remedies. At many places, the knowledgeable adults assist the healers in preparation of medicine for treating patients and in collection of these plants. It is estimated that only 12-20% of the population living in and around the urban area have access to the modern medicine facility and rest have to depend on traditional medicine (Manandhar 1995). MAPs play a vital role in the life support systems of contemporary civilization by serving the purpose of maintaining good health and well being of mankind. But many of these herbs are undocumented and quite poorly understood.

2.3 Research on Medicinal Plants in Nepal

Historically research on medicinal plants began with documenting traditional knowledge into the written texts. Ethnomedicinal survey of various indigenous communities worldwide began during the first half of 20th century and it still continues today with renewed interests. Though natural product from plant was first isolated in pure form early in 1800s (Ji *et al*. 2009), intensive phytochemical and biomedical researches on medicinal plants began only in second half of the 20th century after various technological innovations (Dahanukar *et al.* 2000, Phillipson 2007). In recent time, a large number of institutions from botanical gardens to pharmaceutical companies have been screening ethnomedicinal plants of South America, Africa and Asia in search of sources of potential new drugs (Taylor *et al*. 1995, 1996, Haque *et al.* 2000, Balunas and Kinghorn 2005, Rajbhandary *et al.* 2007, Sharma *et al.* 2008). However, biological studies of medicinal plants such as demography, reproductive biology, ecophysiology (with particular emphasis to plant responses to environmental

changes) and agro-technology are lagging behind in comparison to phytochemical and biomedical researches.

Documentation of ethnomedicinal knowledge is still dominating the researches on MPs in the developing countries. As of December 2003, out of total 590 references on ethnobotany 253 (40%) dealt with MPs (Shrestha *et al.* 2004). Majority (53%) of the references on researches on MPs in Nepal have focused on ethnomedicine and related issues (Ghimire 2008). However, a large number of medicinal plants and ethnic groups are awaiting documentation of their traditional uses. In a study of herbal drugs used by Raute- tribe in far western region of Nepal, Manandhar (1998) reported the use of 32% (15 out of 47 species) of medicinal plants that were not reported earlier. From a survey of a small village in Dolakha district, Shrestha and Dhillion (2003) claimed that about 50% (56 out of total 113) of the ethnomedical remedies they reported were new to ethnobotanical knowledge of Nepal. Similarly, ethnomedicinal uses of about 16 % (18 out of 115 plant species) ethnomedicinal plants used by Chepang communities of Shaktikhor village (Chitwan district) were first reported by Rijal (2008). Out of 1792 plant species (including lichens and fungi) described in 'A Compendium of Medicinal Plants in Nepal' by Baral and Kurmi (2006), ethnomedicinal information of 323 (18.02%) species were completely derived from the documentations from outside Nepal. Despite major effort on ethnomedicinal documentations, aforementioned examples have shown that the probability of finding new medicinal plants and traditional remedies is still high in Nepal.

Dobson (1995) concluded that one out of 125 plant species studied has produced a major drug. Himalayan region has been praised for high diversity in MPs, but pharmaceutical potential of native MPs of this region has not been adequately exploited (Dhar *et al.* 2000). Review of available literatures (Watanabe *et al.* 2005, internet sites PubMed, Scirus, Google and personal collections) revealed that about 400 medicinal and aromatic plants of Nepal have been examined at least once for phytochemical composition (from screening to isolation and characterization of secondary metabolites) and biological activities. However, the number of medicinal plants in Nepal that has been analyzed chemically leading to isolation and characterization of at least single compound did not exceed seventy (Shrestha 2010).

2.4 Ecophysiology

Plant ecophysiology is an experimental science that seeks to describe the physiological mechanisms underlying ecological observations (Larcher 1995). In other words, ecophysiologists, or physiological ecologists, address ecological questions about the controls over the growth, reproduction, survival, abundance, and geographical distribution of plants, as these processes are affected by interactions between plants with their physical, chemical, and biotic environment. Plant ecophysiological research has a fundamental role in advancing the frontier of knowledge essential for a better understanding of plants and their interactions with surrounding environments (El-Sharkawy 2006) for the entire or any period of the life cycle. Plants are frequently exposed to a variety of harsh environmental conditions which negatively affect growth and crop yield. An understanding of the responses of plants to their environment is thus fundamental to minimise the deleterious impact of unfavourable climatic conditions and to manage them for maximum productivity.

Boyer (1982), for instance, argued that water supply affects productivity of trees and annual crops more than all other environmental factors combined. This aspect has been deeply explored, in this issue as for banana, cashew, cassava, coconut, papaya, and tea; however, the development of internal water deficit may be important to some crops such as coffee and mango in order to trigger phenological events such as flower bud release. Decreases in yield induced by low soil water supply may largely be associated with a decline in photosynthetic rates, either by a direct effect of dehydration on the photosynthetic apparatus or by an indirect effect by way of stomatal closure, which restricts CO_2 uptake (Da Matta 2007). In addition to soil water deficits, atmospheric water deficit is also of particular relevance to tropical tree crops. This is due to very low root hydraulic conductivity compared with annuals, which brings about a pronounced effect of transpiration on tree water relations (Da Matta 2003). Limited water supply is one of the most important environmental factors affecting productivity of crops and medicinal plants (Rahman *et al.* 2004). Physiological changes in plants, which occur in response to moisture deficiency decrease photosynthesis and respiration (Hall *et al.* 1990) and as a result overall production of the crop is decreased. For example, the results of Baher *et al.* (2002) showed that greater soil water stress decreased plant height and biomass of *Satureja hortensis*. Colom and Vazzana (2002)

also showed that the number of stems per plant and plant dry mass were negatively related to water stress in *Eragrostis curvula.*

Light is one of the most important environmental factors affecting plant survival, growth, reproduction, and distribution. Light intensity affects photosynthesis, and which in turn, is related to the accumulation of organic matter and biomass. Moreover, to sustain higher photosynthetic capacity or survival, plants modify their morphology and biomass allocation at different light conditions (Den Dubbleden and Oosterbeek 1995). For example, plants grown at low light intensities have higher specific leaf areas (SLA) and leaf area ratios (LAR), and lower biomass and root shoot ratios (R/S) (Lentz1 and Cipollini 1998, Semb 1996). Different species, however, respond differently to light intensity. Light-demanding species are more flexible in both morphology and biomass allocation in response to light change than shade tolerant species (Lortie and Aarssen 1996, Valladares *et al.* 2000). Ryser and Eek (2000) suggested that the differences in adaptive phenotypic plasticity among species may contribute to their different abilities to occupy variable and diverse habitats in the nature. Thus, studies on the plasticity responses of plant species to light environments contribute to our understanding of the ecological mechanism of plant distribution and assist in the development of conservation plans to important plant species.

Nutrient content of soil also affects physiological activity of plants. The nature of soil, associated with soil attributes such as texture, organic matter, pH and bulk density is aptly known to be a potent determinant of plant adaptation and distribution (Epstein 1972). Soil texture is an important index of soil quality. It influences organic matter accumulation (Hassink 1996), distribution of soil N (Hook and Burke 2000) in association with topography and also independently (Raghubanshi 1992), and the dynamics of soil water (Lauenroth and Milchunas 1992) that most frequently limits the biological processes in semi-arid regions.

2.4.1 Effect of Shade on Growth, Yield and Quality

Solar radiation is one of the prime factors governing the growth and yield of crop plants. Jones and McLeod (1990), using 5, 20, 53 and 100 % of daylight, found a higher production and dry matter accumulation in *Sapium sebiferum* when the seedlings were submitted to full sunlight. Dhopte *et al.* (1999) observed that the growth of *Aloe barbadensis* in green poly net house (50 % light intensity) increased by 84 % along

with 81% increase in leaf number and 84% increase in leaf length compared to growth under ambient conditions. Shade increased plant height, number of nodes, mean internodal length and various growth attributes in *Cassia angustifolia* (Vyas and Nein 1999). The leaf growth also increased in terms of number and accumulation of dry matter. The promoting effect was more prominent at 25% shade; however, the impact of further increase in shade level was marginal.Under experimental conditions, 50% shading of *Centella asiatica* resulted in higher yields of asiaticoside and herbage in most of the accessions collected from ecologically different areas whereas in some cases high yield was obtained under full light (Mathur *et al.* 2000). Zingiberaceous spice crops like *Zingiber officinale*, *Curcuma longa* and *Curcuma amada* exhibited better growth and yield in partially shaded situation (25-50%) (Jayachandran and Nair 1998, Nizam and Jayachandran 1997). Alvarenga *et al.* (2003), using 0%, 30, 50, and 70% of solar radiation, found a higher production and dry matter accumulation in *Croton urucurana* when plants submitted to 70% shading.

2.4.2 Effect of Organic Manures on Growth, Yield and Quality

In addition to supplying nutrients, organic manure improves the physical properties of soil by increasing its capacity to absorb and store water, by enhancing aeration and by favouring the activities of lower organisms. Application of organic manure generally increases the growth, yield and quality of crops. *Kaempferia galanga* responded well to organic manuring and gave higher yields with 30 t ha^{-1} of FYM (Thomas *et al*. 1997, 1998). Joy and Thomas (1999) observed that organic manure application enhanced growth, bloom and tuber formation in *Gloriosa superba*. Singh *et al.* (2000) concluded that higher yields of *Plantago ovata* could be achieved by sowing in ridges with the application of organic fertilizer Celrich 23. Kurian *et al.* (2000) reported that the growth, yield and quality of *Piper longum* under differential manurial regimes as intercrop in coconut garden increased with an application of 20 t ha^{-1} of organic manure. Chand *et al.* (2001) reported that yield of essential oil was significantly affected by combined application of manures and fertilizers in *Mentha arvensis.* Significant increase in yield of *Curculigo orchioides* by addition of poultry manure compared to control (without fertilizer) was reported by Joy *et al*. (2005). *Abelmoschus esculentus* responded well to organic manuring and gave higher yields with 20 t ha^{-1} of FYM (Premsekhar and Rajashree 2009).

2.4.3 Effect of Inorganic Fertilizers on Growth, Yield and Quality

Inorganic fertilizers are increasingly used for enhancing crop production, particularly as a source of major nutrients. Though medicinal plants are mostly raised under organic farming, many of them show good response to application of inorganic fertilizer. Maurya *et al.* (1999) observed that 60 kg N ha^{-1} was suitable for higher root yield of *Rauvolfia serpentina*. Tiwari *et al.* (2000a) observed that *Acorus calamus* responded well to N up to 100 kg ha^{-1} and further increase up to 200 kg ha^{-1} did not increase the yields. Kurian *et al.* (2000) reported that phosphorus had a favourable influence on the diosgenin content of *Costus speciosus*. Highest levels of N (150 kg ha^{-1}) and P (90 kg ha^{-1}) and lower levels of K (30 and 60 kg ha^{-1}) recorded higher values for total crude alkaloid in leaf. Singh (2000) reported that application of 50 and 100 kg N ha^{-1} increased oil and artemesinin yields by 26.2 per cent and 40.1 per cent respectively. Singh *et al.* (2000) found that *Plantago ovata* needed 30 kg N and 40 kg P_2O_5 ha^{-1} for better quality husk. However, qualitative analysis of *Mentha* oil by Kattamani *et al.* (2001) found that the application of nitrogen and phosphorus decreased the menthol content and increased the esters and ketones. Nehara *et al.* (2002) recorded that increasing level of phosphorus up to 40 kg P_2O_5 ha^{-1} and potassium up to 45 kg K_2O ha^{-1} significantly increased all the growth characters, yield attributes and yield in *Trigonella foenum graecum* .

2.4.4 Effect of Integrated Nutrient on Growth, Yield and Application

Integrated application of organic manure, inorganic fertilizer and biofertilizer is a more realistic approach in nutrient management of crops. Kurian *et al*. (2000) reported that in *Kaempferia galanga*, integrated application of FYM 20 t ha^{-1} + *Azospirillum* 2.5 kg ha^{-1} + 25 kg N and 50 kg each of K_2O and P_2O_5 + neem cake 1.5 t ha^{-1} + P solubilizer was beneficial for obtaining a consistently higher yield. Harinkhede *et al*. (2000) reported that *Plumbago zeylanica* gave highest dry root yield with 10 t ha^{-1} of FYM and 60:40:30 kg NPK ha^{-1} that resulted in increased root length, number of branches per plant, plant height and root weight per plant. In *Mentha arvensis* herb yield as well as accumulation of major and micronutrients were significantly increased by combined application of manures and fertilizers (Chand *et al*. 2001). Amujoyegbe *et al.* (2007) reported a significant increase in yield and chlorophyll contents of *Zea mays*

and *Sorghum bicolour* in mixture of inorganic fertilizer and poultry manure compared to individual application and in control (without fertilizer). Pirdashti *et al.* (2010) reported that the yield of *Glycine max* in application of 40 Mg ha^{-1} SS (sewage sludge) enriched with half chemical fertilizer increased in comparison to other fertilizer treatments. Integrated nutrient application has a more favourable influence on the growth, yield and quality of crops.

2.4.5 Effect of Moisture on Growth, Yield and Quality

Limited water availability is one of the most important environmental factors affecting productivity of crops and medicinal plants (Rahman *et al.* 2004). Physiological changes in plants, which occur in response to moisture deficiency decrease photosynthesis and respiration (Hall *et al.* 1990) and as a result overall production of the crop is decreased. Greater soil water stress decreased plant height and total fresh and dry weight of *Satureja hortensis* (Baher *et al.* 2002). It was shown that the number of stems per plant and plant biomass were negatively related to water stress in *Eragrostis curvula* (Colom and Vazzana 2002). EI-Saeid (1981) mentioned that in *Phaseolus vulgaris* grown at 90% of field capacity produced higher number of pods relative to those maintained at 54% of field capacity. Furthermore, El-Bettagy and Soliman (1985) studied the effect of water logging for 4 to 8 days on *Lycopersicon esculentum* and found a decrease in plant height, leaf number and chlorophyll content. Rashidi and Seyfi (2007) reported the effect of water stress on crop yield and yield components of *Cucumis melo* where irrigation based on 30% available water deficit (AWD) was found to be more effective irrigation method in improving water use efficiency.

2.4.6 Effect of Soil Texture on Growth, Yield and Quality

The nature of soil, associated with soil attributes such as texture, organic matter, pH and bulk density is aptly known to be a potent determinant of plant adaptation and distribution (Epstein 1972). Soil texture is an important index of soil quality. It influences organic matter accumulation (Hassink 1996), distribution of soil N (Hook and Burke 2000) in association with topography and also independently (Raghubanshi 1992), and the dynamics of soil water (Lauenroth and Milchunas 1992) that most frequently limits the biological processes in semi-arid regions. Travlos and Karamanos (2006) studied the effect of soil texture on growth and yield of the *Tylosema esculentum* plant. They reported well drained light and sandy soils had significant role

on growth and yield of *Tylosema esculentum*. The effect of variation in soil texture on the vegetative and pod characteristics of NH47-4 variety of okra (*Abelmoschus esculentus*) was investigated by Akinyele and Temikotan (2007). They reported a significant role of soil texture on all growth traits and yield of *Abelmoschus esculentus*.

2.5 *Centella asiatica*

Centella asiatica (L.) Urban (Syn. *Hydrocotyle asiatica* L.) of the family Umbelliferae (Apiaceae) is a weakly scented species generally growing in damp, shadowed or swampy areas of savanna and secondary forest clearings in warm climates of both the northern and southern hemisphere such as in parts of India, Sri Lanka, China, Indonesia, Malaysia, Australia, Southern and Central Africa (Verma *et al.*1999, Tiwari *et al.* 2000 b). It is native to Asia and mainly found in India, Nepal, Pakistan and Madagascar but also grows in tropical and equatorial Africa, America, and the tropical regions of the New World (Solet *et al.* 1998). *C. asiatica* reproduces through vegetative and sexual means; however the later mode of reproduction is negligible (Wanker and Tripathi 1993). It is so easily grown that it was once recommended for preventing soil erosion and also for use as a cover crop in tea and rubber plantations (Ng 1998).

2.5.1 Morphological Description

Full morphological description on *Centella asiatica* was given by Solet *et al*. (1998). They illustrated *C. asiatica* as a slender tropical herbaceous plant with crawling stems, propagating vegetatively by runners (stolons), with entire kidney shaped leaves (1-3 cm long, 2-4 cm wide) bearing a crenate margin at the tip of long petioles (5 to 10 times the leaf length). In sunny places petioles are shorter and the petioles and leaves become red due to production of important anthocyanin . Leaves and short peduncled (2-4 cm) inflorescences arise from a rosette near the ground. The umbels are reduced and contain mostly two to four white or pink-purple uniform small flowers (2 mm) consisting of five petals, five free stamens, a greatly reduced calyx, an inferior ovary with two carpels and a stylopodium supporting two styles. The fruit is a dry, flattened schizocarp with two single seeded mericarps each with prominent ridges.

Meanwhile, Solet *et al*. (1998) have also determined three varieties of *C. asiatica* in relation to geographic origin which are correlated with some leaf morphology and chemical composition: (a) *C. asiatica* L. var *typica* has weakly hairs, typically kidney

shaped leaves with well crenulated margins and is found in Southern Asia as far as Madagascar; (b) *C. asiatica* L. var. *abyssinica* shows sub orbicular with softer crenated margins, quite hairy leaves and is present in tropical and equatorial Africa; (c) *C. asiatica* var. *floridana* with leaves longer than wide in shape, is found in America (from Southern United States to Argentina) and in tropical Oceania. Each variety was reported showing several chemical compositions, for example in India, the most frequent type of the variety contains asiaticoside and madecassoside whereas variety *floridana* contains brahmoside and brahminoside.

2.5.2 *In vitro* regeneration

In vitro propagation by tissue culture has been considered as an important tool for conservation and propagation of rare and threatened plants. A successful protocol for the regeneration of callus cultures of *Centella asiatica* was established by Patra *et al.* (1998) in which the stem and leaf explants were cultured on MS media supplemented with 2.0 mg/l kinetin and 4.0 mg/l of a-naphthaleneacetic acid (NAA). These were then regenerated after 4 weeks of subculture using 4.0 mg/l 6-benzyladenine (BA), 2.0 mg/l kinetin (Kn), and 0.25 mg/l NAA and 20 mg/l adenine sulphate.

Centella asiatica multiple shoots were obtained from field-grown plants in MS medium supplemented with 1.0 mg/l BAP within 7 days of culture (Singh *et al.* 1999). Banerjee *et al. (*1999) reported that initial sprouting in *Centella* required the presence of 6-benzyl aminopurine (BAP) (2 mg/l) and indole-3-butyric acid (IBA) (0.1 mg/l); however for multiple shoot induction a higher concentration of BAP (3.0 mg/l) and a lower concentration of NAA (0.05 mg/l) is required. Hossain *et al.* (2000) studied *in vitro* propagation of *C. asiatica*, in which stem node explant of naturally grown plant was used for *in vitro* regeneration of multiple shoots. Various combinations of BAP and NAA in different concentrations were used in the regeneration of multiple shoots and a concentration of 1.0 mg/l BAP and 0.5 mg/l NAA was found superior in the optimum production of multiple shoots. Among the three auxins used in different concentrations, 0.2 mg/l IBA was found effective in the production of roots.

Tiwari *et al.* (2000b) developed a protocol for rapid and large-scale *in vitro* clonal propagation of the valuable medicinal herb *Centella asiatica* by enhanced auxiliary bud proliferation in nodal segments isolated from mature plants. Subculturing of nodal segments harvested from the *in vitro* derived axenic shoots on the multiplication

medium enabled continuous production of healthy shoots with similar frequency.MS medium supplemented with 6.7 –M BA and 2.88-M indole acetic acid (IAA) was found most suitable for shoot elongation. Rooting was highest (90%) on full-strength MS medium containing 2.46 -M IBA. Nath and Buragohain (2003) developed a method for rapid clonal propagation for *C. asiatica* by shoot tip (2-3 cm long) culture. The shoot tips isolated from mature plants were inoculated on MS medium incorporated with BA alone or in combination with NAA and kinetin. The optimum number of shoots (3.38) with optimum number of leaves per shoot (4.25) was attained on MS medium supplemented with 4.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA. On transferring the micro shoots on full strength MS medium supplemented with various concentrations of IBA (1.0-3.0 mgl^{-1}) and NAA (0.5-2.0 mgl^{-1}), profuse rooting (46.8 per shoot) was obtained in MS basal medium with 2.0 mgl^{-1} IBA with root length of 19.7 cm. Paramageetham *et al.* (2004) produced abundant somatic embryos from leaf segments excised from *C. asiatica* when cultured on MS medium with 9.29 μM kinetin in combination with 2.26 μM 2-4-dichlorophenoxyacetic acid (2, 4-D).

Sivakumar *et al.* (2006) developed protocol for rapid clonal propagation of *Centella asiatica*, using shoot tip culture. High frequency bud break (88 %) and multiple shoot formation (16.8 shoots/shoot tip) were induced from a shoot tip segment, which was cultured on MS medium supplemented with 6-benzylaminopurine (BAP) (17.76 µM) and gibberillic acid (GA_3) (1.44 µM). Half-strength Murashige and Skoog (MS) medium supplemented with naphthalen acetic acid (NAA) (10.74 µM) induced the maximum (27.66) number of roots. Karthikeyan *et al.* (2009) described rapid clonal propagation of *C. asiatica* from single nodal explants using Murashige and Skoog's (MS) medium containing different concentrations and combinations of 6-benzylaminopurine (BAP) and Kinetin (Kn).

2.5.3 Chemical Constituents

Phytochemical analysis by different researchers revealed the presence of triterpenes, alkaloids, volatile compounds and amino acids in *Centella asiatica.* The main components of *C. asiatica* are triterpenes asiaticoside and madecassoside and their aglycones asiatic and madecassic acid. Other glycosides reported include indocentelloside bhramoside, bhraminoside, theankuniside and isotheankuniside (Jamil *et al*. 2007). Inamdar *et al.* (1996) determined the biologically active constituents as

asiatic acid, madecassic acid and their respective glycosides, asiaticoside and madecassoside in *Centella asiatica* by high performance liquid chromatographic method. Meanwhile, Banerjee *et al.* (1999) also reported that the plant contains several other triterpenes such as centelloside, bhramic acid as well as the alkaloid hydrocotylin. Shukla *et al.* (1999) separated a new ursane triterpenoid from *C.asiatica* and exhibited its dose dependent growth inhibitory activity against larvae of *Spilarctia oblique*. Gupta *et al.* (1999a) also reported variable asiaticoside contents in five lines of *C. asiatica* collected from a field trial in India, mean levels varying from 0.42 to 1.17%. Matsuda *et al.* (2001) isolated a new olean 1-3-ene triterpene (centella sapogenol A) and its oligoglycoside from *C. asiatica* cultivated in Vietnam, and two new ursane type triterpene oligoglycosides (centellasaponins B and C), and an oleanane type triterpene oligoglycoside (centellasaponin D) from a genotype cultivated in Sri Lanka. Kuroda *et al.* (2001) also separated five new triterpene glycosides from the aerial parts of *C. asiatica* and none of these saponins revealed significant cytotoxicity. Moreover, Jiang *et al*. (2005) identified four new triterpenoid glycosides named asiaticoside C, D, E, and F from the butanol fraction of *C asiatica*. Randriamampionona *et al.* (2007) reported high amount of asiaticoside content (2.67 to 6.42 %) in *C. asiatica* of Madagascar.

Besides triterpenes, *C. asiatica* contains other primary and secondary products such as, volatile oil of a terpene acetate ,camphor, cineole, glycerides of some fatty acids, plant sterols (campesterol, stigmasterol, sitosterol), polyacetylene compounds, flavonoids (kaempferol, quercetin), myo-inositol (glycoside from the flavonoids), sugars, the bitter principle vellarin and resins (Jamil *et al.* 2007). Apart from that, the presence of numerous caffeic acid derivatives as well as mono- and sesquiterpenes (β-caryophyllene, trans β-farnesence, germacrene –D) (Asakawa *et al*. 1982) have also been reported. The major constituents of the volatiles as determined by Jayatilake and MacLeod (1987) in *C. asiatica* grown in Sri Lanka are shown in Table 2.1.

Qin *et al.* (1998) identified 45 volatile components by GC/MS from *C. asiatica* of which the main constituents were caryophyllene, farnesol, and elemene. Subsequently, they found the antidepressant effect of volatile oil extract in mouse. Ng (1998) analyzed amino acids in different parts of *C. asiatica* and found that the concentrations of glutamate and serine is higher than other amino acids in leaf, petiole and stolon. Root is

also rich in amino acids; especially the aspartate, glutamate, serine, threonine, alanine, lysine, histidine and amino butyrate.

Table 2.1 Volatile compounds of *Centella asiatica* (Source: Jayatilake and MacLeod 1987).

Components	Relative abundance (%)
α-pinene	3
β –pinene	3
Camphene	1
Myrcene	3
α –terpinene	5
γ-terpinene	5
α-copaene	14
Calarene	4
β-Carryophyllene	12
Trans- β-farnesene	5
α-humulene	9
δ-cadinene	2
δ-cadinol	1

Ng (1998) also revealed that the total essential and non essential amino acids were 7.6 and 7.2 g per 100g of dry sample, respectively. The most abundant amino acids were aspartic acid (2.01±0.06 g/100 g DW), glutamic acid (1.86 ±0.06 g/100 g DW), leucine (1.36±0.01 g/100 g DW) and valine (1.04 ± 0.08 g/100 g DW) (Table 2.2). Analyses of the volatile fraction of this medicinal plant, growing in South Africa, revealed 11 monoterpenoid hydrocarbons, 9 oxygenated monoterpenoids, 14 sesquiterpenoid hydrocarbons, 5 oxygenated sesquiterpenoids and 1 sulfide sesquiterpenoid (Oyedeji and Afolayan 2005). The predominant constitutes were α-humulene, β caryophyllene and bicyclogermacrene. The volatile extract exhibited a broad spectrum of antibacterial activities against both Gram positive and Gram negative organisms.

2.5.4 Therapeutic Application

C. asiatica and its preparations have been used in the folk medicine in several cultures mainly for the treatment of various nervous and skin disorders and also as tonic, diuretic and antihypertensive. From the available literature it can be concluded that its major medicinal properties are due to the triterpenes. For instance, Youshinari *et al.* (1982) disclosed that bhramic acid, which is another biologically active triterpenoid present in *C. asiatica* has therapeutic value in ulcerations, extensive wounds and eczemas.

2.5.4.1 Skin disorder and wound healing

Clinical trials have also shown that extracts of *C. asiatica* heal wounds, burns and ulcerous abnormalities of the skin such as chapping, grazing, insect stings, sun burn and other light, small burns, cicatrisation after surgery in gynecology, ophthalmology (cornea lesions) or eardrum lesions (Vogel *et al.* 1990), cure stomach and duodenal ulcers and are effective in the treatment of leprosy, lupus as well as scleroderma (Kartnig 1988). Its wound healing effects is due to its up-regulation of human collagen I expression (Bonte *et al.* 1994) and an increase in tensile strength of wounds (Suguna *et al*. 1996). Meanwhile study by Cheng and Koo (2000) suggested that the anti-ulcer mechanisms of *C. asiatica* extract may be due to its strengthening action on gastric mucosal lining and the suppression of damaging effects of free radicals.

The commercial drug preparations of *C. asiatica* are administered worldwide particularly in West Germany and France in many formulations (Kartnig 1988), mainly as an ointment whilst infusion or poultices of *C. asiatica* have been used in Europe since the 18^{th} century for the treatment of lesions of leprosy (Kartnig 1988). A drug derived from *C. asiatica* has been developed in the European Pharmacopeia, under the name of 'Titrated Extract from *C. asiatica* (TECA)'. It is a reconstituted mixture of three triterpenes purified from the plant, asiatic acid, madecassic acid and asiaticoside (Maquart *et al.* 1999).

2.5.4.2 Blood circulation, respiratory system and antihypertensive

Centella asiatica is used as an antipyretic, detoxicant (blood purifier) and diuretic agent in Chinese traditional medicine. The leaves of *C. asiatica* also prescribed in curing leucorrhoea and toxic fever in Chinese community. Other commonly cited uses include the treatment and alleviation of respiratory complaints such as relief from

congestion due to cold, asthma,bronchitis, tuberculosis (Tiwari *et al*. 2000 b), kidney trouble and urethritis (Jaganath and Ng 1999) besides improving blood circulation throughout the body by strengthening the veins and capillaries (Kartnig 1988).

Table 2.2 Free and total amino acids in *Centella asiatica* (Source: Ng 1998)

Amino acid	Free amino acid (mg/100 g FW)	Total amino acid (g/100 g DW)
Aspartic acid	56.47±3.36	2.01±0.06
Glutamic acid	58.10±7.42	1.86±0.06
Serine	3.78±0.30	0.75±0.02
Asparagine	12.55±2.6	-
Glycine	0.45±0.07	0.78±0.08
Glutamine	6.36±0.60	-
Histidine	2.17±0.38	0.29±0.01
Arginine	1.92±0.21	0.80±0.03
Threonine	6.25±0.7	0.78±0.02
Alanine	20.74±3.03	0.84±0.09
Proline	1.04±0.34	0.80±0.08
Tyrosine	42.64±6.27	0.54±0.01
Valine	7.24±0.83	1.04±0.08
Methionine	0.80±0.13	0.25±0.02
Cysteine	Trace	0.16±0.01
Isoleucine	3.74±0.23	0.69±0.07
Leucine	4.26±0.30	1.36±0.01
Phenylalanine	9.50±0.53	0.92±0.02
Tryptophan	Trace	Not detected
Lysine	3.90±0.20	0.88±0.02
Essential amino acids	82.42	7.55
Non essential amino acid	159.49	7.20
Total	241.91	14.75

C. asiatica is also reported to possess anti-epileptic activity and is sometimes quoted in the treatment of phlebitis as well as leg cramps, swelling of legs and heaviness or tingling in the legs (Michael 2003). In the course of pharmacological studies, the plant showed central nervous system depressant activity (Sakina and Dandiya 1990). Juice prepared from fresh *C. asiatica* is consumed as a cooling drink and for treating hypertension by the Chinese (Ng 1998). According to Mahato and Chaudhary (2005), about four teaspoonfuls of leaf juice (juice obtained by squeezing 50 leaves between

palms of hands) is taken orally in the morning for 2-3 weeks for its alleged cooling property to body and stomach. About 15 ml of leaf juice mixed with about 5 g mixture of 'Alainchee', Pipla', 'Jeethimadhu' and 'Gund 'is given 2-3 times a day to cure cough. Leaf paste prepared with cow's urine is applied on nose and forehead to cure sinusitis 'Pinas' by local people of western Nepal (Panthi and Chaudhary 2006).

2.5.4.3 Mental enhancement

Indians used dried powder of *Centella asiatica* as a tonic for the brain (Ng 1998). Nalini *et al.* (1992) have shown that its fresh leaf juice improves passive avoidance task in rats and it is thought to enhance mental concentration, treat mental fatigue, anxiety (Goh *et al.* 1995) promote relation as well as to improve learning capacity in rat (Rao *et al.* 1999). The whole plant of *C. asiatica* has been shown to be beneficial in improving memory (Gupta *et al*. 2003) and is also reported to improve the general ability of mentally retarded children (Veerendra Kumar and Gupta 2002). In addition, it has also been widely sold as a body strengthener, revitalizer that can promote longevity and a weak sensitizer (Hausen 1993). Meanwhile, studies by Shobi and Goel (2001) suggested that *C. asiatica* could be useful in preventing radiation induced behavioral changes during clinical radiotherapy.

2.5.4.4 Cytotoxicity and antitumor properties

Babu *et al*. (1995) revealed the cytotoxicity and anti-tumor properties in *Centella asiatica*. Methanolic extract of *C. asiatica* was found to have inhibitory effect on the biosynthetic activity of fibroblast cells (Shukla *et al*.1999).Triterpene glycosides in *C. asiatica* have been identified as having oncogenic activity and oral administration of its extract retarded the development of solid and ascites tumor and increases the life span of tumor bearing mice (Babu *et al*.1995).

2.5.4.5 Cosmetic application

Centella asiatica has also been used widely in cosmetic which include the formation of lipids and proteins for healthy skin, anti-cellulites, anti-wrinkle for eyes and facial, skin tightening properties, skin regenerative, treatment of acne induced blemishes, rapid renewal of supporting fiber network and to boost immuno-depressed skin (Michael 2003).

2.5.4.6 Other applications

Along with the use of *Centella asiatica* in medicine, the plant is also taken as a vegetable as its leaves are particularly rich in carotenoids, vitamin B and C (Ng 1998). The leaves of *C. asiatica* are used as vegetable (in curries and salads) in Kerala, India. In Malaysia, it is cooked as vegetable or eaten as raw as salads and eaten with rice. It is also commonly used in porridge for feeding pre-school children in Sri Lanka in combating nutritional deficiencies (Zainol *et al.* 2003).

2.6 Triterpenes in *Centella asiatica*

Asiatic acid, madecassic acid and asiaticoside (Fig 2.1) that belong to *β*-amyrin ursolic acid group are the major triterpenoids found in *Centella asiatica*. Asiatic acid ($C_{30}H_{48}O_5$) is the 2, 3, 23-trihydroxy -12-en-28-oic acid. Madecassic acid is the 6-hydroxylated asiatic acid and it differs from asiatic acid by one hydroxyl function (Laugel *et al*. 1998). Asiaticoside results from an esterification of carboxylic function by three glycosyl residues (Laugel *et al*. 1998). Asiaticoside is trisaccharide (*O-α-L*-rahmnopyranosyl (1-4), *O-B*-glucopyranosyl (1-6), *O-B-D*-glucopyranose ester of asiatic acid (Solet *et al*. 1998).

Other triterpenes have also been described, such as madecassoside (Fig 2.1), which is the same trisaccharride as in asiaticoside esterifying madecassic acid, madasiatic acid (a 2,3-6 trihydroxylated isomer), brahmic acid (a 2,3,6,23 tetrahydroxy isomer of madecassic acid), brahmoside, bhraminoside (respectively a glycoside and an ester glycoside of brahmic acid both with a rhamnosyl glycosyl arabinosyl–trisaccharide), thankunoside, isothankunic acid, isothankuniside, (a 3,5,6,23) tetrahydroxy isomer esterified with a disaccharide of glucose and rhamnose), centellic and centoic acids ($C_{30}H_{48}O_6$), as well as centelloside (a polysaccharide ester of centellic acid containing 10 glucose and 2 fructose).

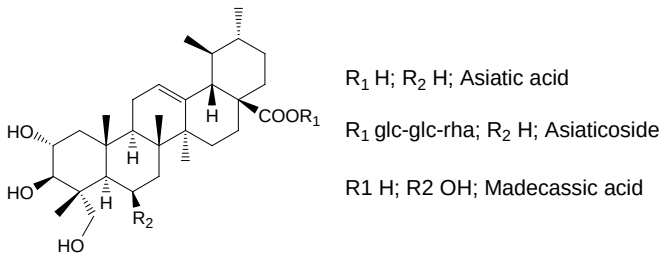


Fig 2.1 Structure of triterpenes of *Centella asiatica.*

2.7 Flavonoids in *Centella asiatica*

Besides triterpenoids, *Centella asiatica* also contain numerous flavonoids, including quercetin and kaempferol, catechin, rutin and naringin, as a major part of the total phenolic contents, some of which are major contributors in particular to the antioxidative activity of *C. asiatica* (Zainol *et al.* 2003). Based on the hypothesis of free radical mediated toxicity in oxidative stress process and depending on its antioxidant properties, *C. asiatica* has been recently indicated to show antilipid peroxidative and free radical scavenging activities (Wong *et al.* 2006). In addition, Matsuda *et al.* (2001) isolated a flavonol, petuletin, and kaempferol 3 *O-β*-D glucuronide from the aerial parts of *C. asiatica* cultivated in Vietnam, both of which exhibited potent inhibitory activity on aldose reductase in rats. Bioflavonoids of *C. asiatica* have ever been exhibited to be efficacious in venous insufficiency, probably due to their actions on mucopolysaccharide metabolism (Matsuda *et al.* 2001).

2.8 Genetic Diversity

The polymorphism in the marker can be detected at three levels: phenotype (morphological), differences in proteins (biochemical), or differences in the nucleotide sequence of DNA (molecular). Morphological markers generally correspond to the qualitative traits that can be scored visually. Isozymes are used as biochemical markers. Isozymes are different molecular forms of the same enzyme that catalyze the same reaction. A molecular marker is a DNA sequence that is readily detected and whose inheritance can easily be detected (Chawla 2004).

Traditionally, diversity has been assessed by measuring variation in phenotypic traits such as flower color, growth habit or quantitative agronomic traits such as yield potential and stress tolerance. This approach, however, has certain limitations; genetic information provided by morphological characters is often limited and expression of quantitative traits is subjected to strong environmental influence. Biochemical methods based on seed protein and enzyme proteins have been useful in analysis of genetic diversity as they reveal differences between seed storage proteins or enzymes encoded by different alleles at one (allozymes) or more gene loci (isozymes). Use of biochemical methods eliminates the environmental influences; however, their usefulness is limited due to their inability to detect low levels of variation. DNA-based

techniques introduced over the past two decades have potential to identify polymorphisms represented by differences in DNA sequences. These methods are being used as complementary strategies to traditional approaches for assessment of genetic diversity. The major advantage being that they analyze the variation at the DNA level itself, excluding all environmental influences. The analysis can be performed at any growth stage using any plant part and it requires only small amounts of material. Following the advances in molecular biology in the last decade, a variety of different methods have been developed for analysis of genetic diversity (Puchooa and Venkatasamy 2005).

Major molecular markers used in genetic study are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNAs (RAPDs), sequence characterized amplified regions (SCARS), simple sequence repeats (SSRs) or microsatellite, sequence tagged sites (STS), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeat amplification (ISA), cleaved amplified length polymorphisms (CAPS) and amplicon length polymorphisms (ALPs). RFLP is hybridization based molecular marker while the remaining are PCR based marker. Each of these markers has some advantages and disadvantages. So, molecular marker should be chosen depending on the intended application, convenience and cost involved (Gupta *et al*. 1999b).

Mathur *et al.* (1999) highlighted two distinct morphotypes of *Centella asiatica* collected from 16 different locations of India by morphological characters. Padmalatha and Prasad (2008) studied genetic diversity of Indian *C. asiatica* using RAPD markers and highlighted the 87% polymorphism among the populations. Ruan *et al.* (2008) also studied molecular characters of Chinese *C. asiatica* with RAPD technology and reported high homogeneity in several samples from different habitats.

Although *Centella asiatica* is a common and widely used medicinal plant in Nepal, there has not been any genetic study in this plant as of yet. In order to conserve genetic resources for plant improvement, it is necessary to preserve, maintain and document genetic diversity (Lane *et al.* 2000). The assessment of genetic variability in natural populations can provide new insights into the evolutionary history and phylogenetic relationships of *C. asiatica*, and can address the cultivation practices (Vaughan 1989). The better understanding of genetic variation at the intraspecific level help in

identifying superior genotype(s) for crop improvement as well as to evolve strategies for the effective *in situ* and *ex situ* conservation programmes, such empirical determination of genetic diversity can be obtained by evaluating morphological, physiological and biochemical traits.

In conclusion, *Centella asiatica* is important for primary healthcare in traditional medicine in Nepal. Quantitative data on distribution, abundance and suitable habitat for growth of the *C. asiatica* is lacking. More than seventy secondary metabolites were isolated from *C. asiatica* collected from neighbouring countries; quantitative variation of triterpene of *C. asiatica* from India, Malayasia and Madagascar has already been reported. However, there was not a single work published on phytochemical analysis of this plant collected from Nepal .

3. STUDY AREA

3.1 Physiography

Nepal (26°22' to 30°27' N and 80°04' to 88° 12' E) lies in the central Himalayan zone. Elevation of country ranges from 60 (Kechana, Jhapa) to 8848 m (Mt Everest). The country occupies a location between China to the north and India to the south, east and west. The average distance between east-west is 885 km and between north-south is between 145-248 km (Kansakar *et al.* 2004). Both eastern and western Himalayan elements are present here. The total area occupied by this country is 147,181 km^2.

Varied climatic and edaphic factors act effectively to bring about significant changes in the ecosystems and habitat types in Nepal. Botanists of British Museum, based upon their extensive researches, have estimated the occurrence of 7000 species of flora in Nepal. Press *et al*. (2000) mentioned the existence of 6,500 species of flowering plants in this country.The published record of the Nepal Fourth National Report to Convention on Biological Diversity (MOFSC 2009) estimates the occurrence of about 6417 species of vascular plants (of which 534 are pteridophytes, 27 gymnosperms and 5856 angiosperms).

Nepal has been divided horizontally into five physiognomic zones: Terai, Siwalik, Mahabharat, Greater-Himalaya and Trans-Himalaya (Hagen 1969, Upreti 1999). Each zone represents a definite elevation range. Similarly, the vegetation of Nepal has been classified into 8 elevational zones: Tropical, Sub-tropical, Lower temperate, Temperate, Sub-alpine, Open low-alpine, Mid-alpine and Nival zones (Stainton 1972, Miehe 1989).

For the present study, the classification based on climate, flora, and ecology of Nepal proposed by Stearn (1960) was followed that categorize the country into three major regions viz. west (Longitude 80^0 40'-83^0 0'E), central (Longitude 83^0 0'-86^0 30'E) and east (Longitude 86^0 30'-88^0 12' E). Banerjii (1963) also classified this country into three main divisions. His floristic divisions are based upon the three major rivers system like Karnali, Gandaki and Koshi.

3.2 Study Areas/Collection sites

Study on the distribution of *Centella asiatica* was done from terai plains to mid hills covering tropical to lower temperate region of Nepal. Varied physiographical

conditions and potentiality of plant available areas were the basic parameters considered for site selection. Collection sites with geographical location are shown in Table 3.1. Distribution and location of sampling sites for the present research are shown in Fig 3.1.

Three different habitats from different locations were selected for study: **1. Partially shaded site** was comparatively undisturbed and densely vegetated area where grazing was prohibited, but other biological activities like cutting and clearing of vegetation was carried out. This site was shaded by tree and shrub like *Pinus roxburghii, Prunus*, *Rubus*, *Populus,* etc. Associated species were *Chromolaena adenophora, Cyanodon dactylon, Setaria* sp., *Geranium* sp. etc. **2. Open grassland** where grazing pressure by cows and buffaloes was high all round the year. This site was open and received full sunlight. The associated species of *C. asiatica* at this site were *Cyanodon dactylon*, *Parthenium hysterophorus, Breea arvensis, Paspalum* sp. etc. **3. Open agricultural land** in fallow period, which was open area, received full sunlight and vegetation coverage of associated species was sparse **.**There was frequent grazing by cows but the grazing pressure was lower than in open grassland**.** Associated species were *Bidens pilosa*, *Cyanodon dactylon*, *Hydrocotyle* sp., *Setaria* sp., etc.

3.3 Climatological Record of some districts

The study area lies in tropical, sub tropical to lower temperate region of Nepal. In the Terai, winter temperature is in between 22-25°C while summer temperature exceeds 35°C. At the foot of the Himalayas, temperature lies between 12-15°C. The climate in the study area is generally dry in October to April, warm in April to June and rainy season starts in June and ends in September. Much of the precipitation comes in the form of summer monsoon rain both from the Bay of Bengal and the Arabian Sea which prevail from mid of June to mid of September. Monsoon conditions begin to affect the eastern end of the country early in June and gradually spread westwards. The eastern region of country receives higher rainfall than western region (Singh 1999). The southern sides of the Mahabharat and the Himalayas receive higher amount of rainfall.

The ombrothermic graphs of the climatological data (mean temperature in ºC and rainfall in mm) of four districts representing three different ecological regions and one Kathmandu district where transplantation experiments were carried out are presented in Fig.3.2.

Transplantation experiments and phenological patterns of *Centella asiatica* were examined at Kathmandu. The Kathmandu valley (85º30' E to 85º 40' and 27º55' N to 27º35' N alt. 1350 m asl) is roughly elliptical in outline with approximately 339 km^2 area. It lies in tropical region of central Nepal with mean temperature from 10 to about 25°C in different months (Figure 3.2). The area receives monsoon rain from June to September, which accounts about 80% of the total annual rainfall (2244 mm). Rest of the months are dry with few showers of winter rain. The valley is surrounded on all sides by hills and mountains (maximum elevation 2720m Shivapuri hill).The soil texture in valley varies from loamy to clay.

Table 3.1 Collection sites and habitats of *Centella asiatica.*

SN	District	Locality	Elevation (masl)	Geographical location	Eco-region**	Habitat	Landuse
1	Ilam*	Phidim	1250 a	26º 72.80' N 87° 90.32´ E	E	Open grassland, Fallow agricultural land	Fallow land, grazing by cattle
2	Jhapa*	Bhadrapur	85	26º 20'-26º30'N 87º39'-88º12'E	E	Fallowagricultura land	Grazing of cattle
3	Sunsari*	Inaruwa	96	26º23'-26º55'N 87º05'E	E	Open Grassland, Shady grassland	Cattle grazing was prohibited
4	Dhankuta*	Hille	1800a	26º 77.70' N 87º 3.54' E	E	Open grassland, Shady grassland	Cattle grazing was prohibited
5	Makwanpur*	Hetauda	650a	27º 37.5' N 85º 0.32' E	C	Open grassland	Fallow land, grazing by cattle
6	Makwanpur	Daman	2300	27º 29.14' N 85º 10.05' E	C	Open grassland	Grazing of cattle
7	Chitwan*	Gauriganj	250	27º21'-27º52'N 83º54'-84º48'E	C	Open Grassland, Fallow agricultural land	Cattle grazing was prohibited
8	Gorkha*	Palungtar	600	28º 01.39' N 84º 38.74' E	C	Open and shady grassland, Fallow agricultural land,	Fallow land, grazing by cattle
9	Lamjung	Way to Besisahar	740	28º 14.57' N 84º 2.66' E	C	Shady grassland	Cattle grazing was prohibited
10	Lalitpur*	Godavari	1550	27º 35.72' N 85º 22.70' E	C	Open grassland	Grazing by cattle
11	Kathmandu*	Kirtipur	1350	27º 40.20' N 85º 17.32' E	C	Open and shady grassland, Agricultural fallow land	Grazing by cattle

Contd…….

12	Kathmandu*	Matatirtha	1400	27º 40.54' N 85º 14.34' E	C	Shady grassland, Open agricultural fallow land	Fallow land, grazing by cattle
13	Kaski*	Pokhara	850	28º 16.99' N 83º 9.33' E	C	Open grass land	Fallow land, grazing by cattle
14	Kaski	Dhampus	1800b	28º 18.012' N 83º 51.13' E	C	Shady grassland, Agricultural fallow land	Cattle grazing was prohibited in shady habitat but not in open grassland
15	Pyuthan	Ampchaur	1250b	28º 2.28' N 82º 7.99' E	W	Open grassland, Shady grassland	Cattle grazing was prohibited in shady habitat but not in open grassland
16	Dang*	Lamahi	150a	27º 9.5' N 82º 4.13' E	W	Open grassland	Fallow land, grazing by cattle
17	Surkhet*	Birendranagar	650b	28º 6.25' N 81º 5.99' E	W	Shady grassland	Cattle grazing was prohibited
18	Banke*	Kohalpur	155	28º 0.73' N 81º 3.99' E	W	Open agricultural fallow land	Fallow land, grazing by cattle
19	Bardiya*	Magaragadi	150b	28º 12.73' N 81º 36.90' E	W	Shady grassland, Open agricultural fallowland	Cattle grazing was prohibited
20	Kailali	Dhangadi	130	28º 7.31' N 80º 2.25' E	W	Open grassland	Fallow land, grazing by cattle
21	Kanchanpur*	Mahendranagar	120	29º 0.50' N 80º 2.25' E	W	Open grassland	Fallow land, grazing by cattle

*Seed collection sites; ** Ecoregions of Nepal: E – Eastern Nepal, C – Central Nepal, and W – Western Nepal

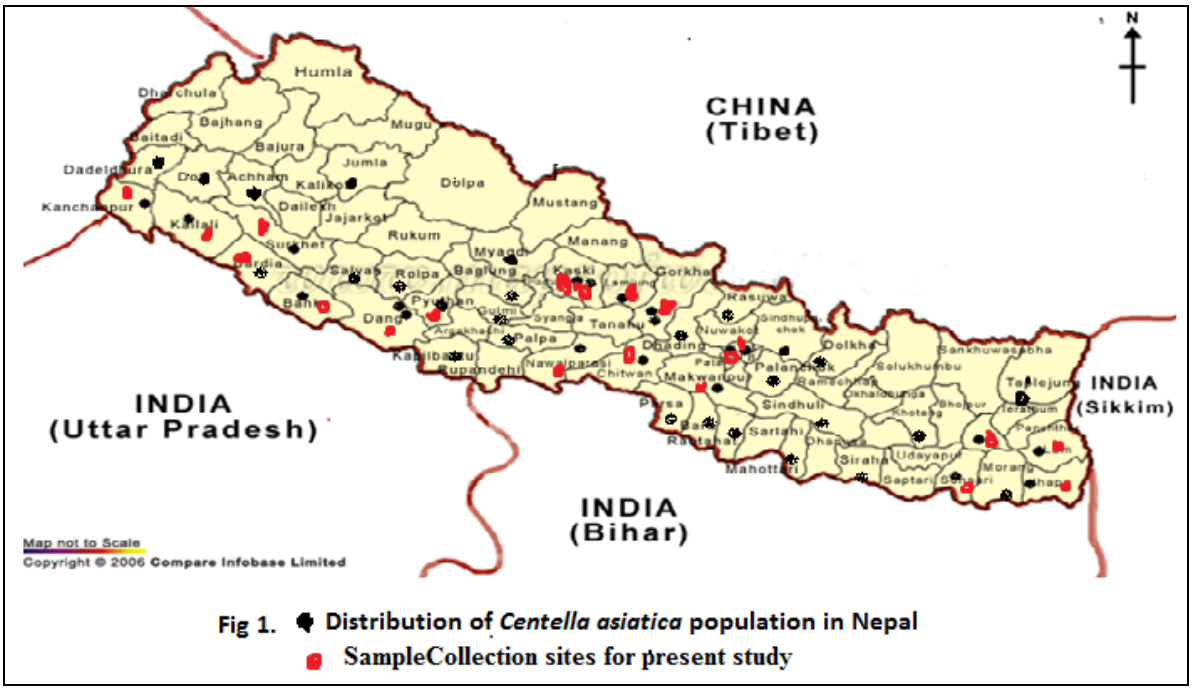


Fig 3.1 Map of Nepal showing locations of sampling sites for the present research

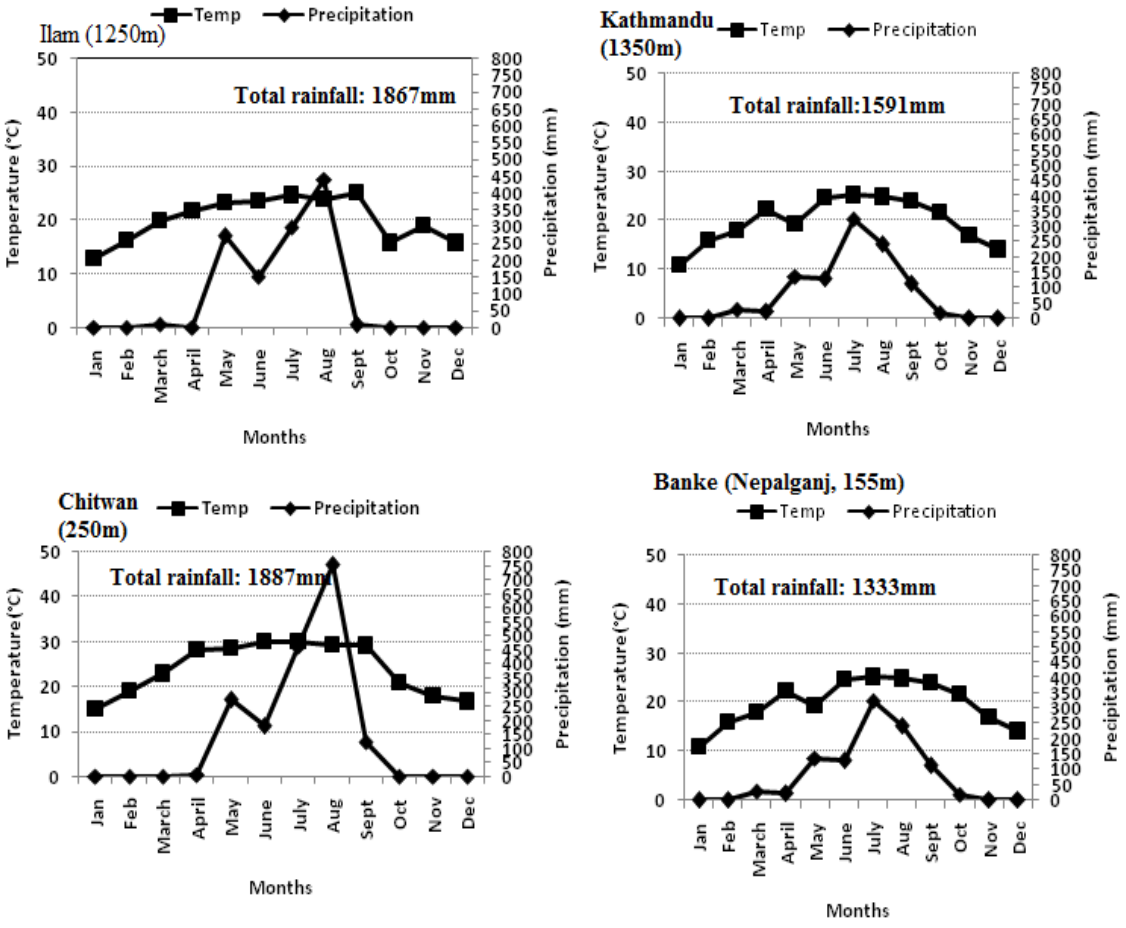


Fig. 3.2 Average monthly mean temperature and precipitation of Kathmandu, Ilam, Banke (Nepalganj) and Chitwan (Bharatpur) from 1999-2008). Source: Department of Hydrology and Meteorology, Government of Nepal, Kathmandu

4. MATERIALS AND METHODS

4.1 Study Species

Centella asiatica (L.) Urban (Synonyms : *Hydrocotyle asiatica* L., Family: Apiaceae (Plate 1) is called 'Ghod tapre' in Nepali; 'Kholchaghayan' in Newari, 'Mandukaparni' in Sanskrit, 'Toprejhar' in Gurung, 'Ghortapre' in Tamang; Water pennywort and Indian pennywort in English; 'Thankuni' in Bengali and 'Brahma-manduki', 'Khulakhudi' in Hindi.

It is a small creeping perennial herb. The stems are slender, creeping stolons, green to reddish green in color, interconnecting one plant to another; heart or kidney shaped leaves emerging alternately in clusters at the node; petiole usually 5 to 13 cm, sometimes longer than the lamina, which is 10 to 40 mm long and 20 to 40 mm, sometimes up to 70 mm, wide. The runners lie along the ground and leaves with their scalloped edges rise above on long reddish petioles. The rootstock consists of creamish rhizome growing vertically down. Inflorescence is auxiliary, simple umbel, peduncle 0.5 - 0.8 cm long, 2-5 flowered, ovate, membranous, and persistent. Flower minute, 0.3 cm in diameter, hermaphrodite, actinomorphic, epigynous, pink, calyx-teeth obsolete, petals 5, ovate, entire, imbricate, stamens 5, filaments short, anther bilobed, dorsifixed; pollinated by insects, self fertile. Ovary is inferior, 2-celled, with one anatropous ovule in each loculus. Styles 2-from the base, filiform, fruit about 0.3 cm long, laterally flattened, depressed ovate-globose, slightly pubescent when young, soon glabrate (DPR 1986). Whole plant is medicinally important.

The stomata are anisocytic (Shah and Abraham 1981) on both surfaces of the leaf, with mostly rubiaceous type. Palisade cells differentiated into two layers of cells, spongy parenchyma of about three layers of cells with many intercellular spaces, some with crystals of calcium oxalate; midrib region shows 2 or 3 layers of parenchymatous cells without chloroplastids; petiole shows epidermis with thickened inner walls; collenchyma of 2 or 3 layers of cells; a broad zone of parenchyma; seven vascular bundles within parenchymatous zone, two in projecting arms and five forming the central strand. Some parenchymatous cells contain crystals of calcium oxalate. Fruits, epidermis of polygonal cells, trichomes similar to the leaves, sheets of elongated

parquetry layer cells, bundles of narrow annular vessels, and parenchymatous cells contain single large prisms of calcium oxalate (Anonymous 1953). The diploid (2n) chromosome number is variable; it is 18, 22, or 33 (Joshi and Joshi 2001).

4.2 Field Sampling

Field observations and samplings were done between 2006 and 2008. Preliminary information on habitat range of *Centella asiatica* was obtained by reviewing literature (Malla *et al.* 1997, Press *et al.* 2000, Joshi and Joshi 2001). Then potential available sites were visited for sampling. Twenty one different populations of *Centella asiatica* were selected representing three different ecological regions of Nepal for the study of soil nutrients, population density and leaf nutrient. Out of these 21 populations, two populations viz. Matatirtha and Kirtipur were selected for the study of reproductive biology.

4.2.1 Population Density, Soil Nutrients and Plant Biomass

In each population, 2-5 plots of 10 m × 10 m size were subjectively choosen to represent the patches with relatively high density of *Centella asiatica.* In each plot, 10 quadrats (1 m × 1 m) were sampled randomly. Altogether 54 plots (10 m × 10 m size) from different eco-regions of Nepal representing three different habitats were sampled; i.e 18 plots each (6 plots each from open agricultural land , open grassland and shaded grassland) from eastern ,central and western Nepal . Thus altogether 18 plots each from open agricultural land, open grassland and shaded grassland were sampled. Total number of individuals of *C. asiatica* was recorded from sampling plots. Because of the rosette habit, each adult with a rosette of leaves and a root system was considered an individual. Ramet density was calculated in number per square meter (pl/ m^2). To determine aboveground biomass, all individuals of *C. asiatica* were collected from a single most densely populated quadrat of the large (10 m× 10 m) plot. So altogether biomass of 54 quadrats was measured.

From each site thirty leaf samples were collected (each from a single ramet) randomly from each plot to determine leaf nitrogen content. The leaves collected were green and least damaged. Collected leaf samples were air dried under shade for seven-eight days and sealed in zipper plastic bags till analysis. From the rooting depth (5-10 cm) of the plant about 200 g soil samples were also collected from each quadrat to determine soil

pH, organic carbon and nitrogen. Altogether 540 soil samples were collected for analysis.Soon after collection, soil samples were air dried under shade.

4.2.2 Growth and Reproductive Traits

During field sampling, individual plants were also collected to measure growth and reproductive traits. Healthy and undamaged 50-60 individuals of *Centella asiatica* were completely uprooted to determine number of leaves per ramet, number of primary branches, stolon length, petiole length, number of flowers borne by individual plant.

Ninety mature leaves per population were measured for leaf length (LL), leaf width (LW), petiole length (PL), leaf area (LA), dry weight of leaf (LDW) and specific leaf area (SLA). Length, width and petiole length were measured in fresh leaves. Then these leaves were oven dried (60ºC, 48 h) and mass of each leaf was weighed in electric balance (0.001 g). Length and width of leaves were measured and multiplied by conversion factor following Zobel *et al.* (1987) to determine leaf area. SLA was calculated as the ratio of projected leaf area and dry mass. From each population, 30 samples of leaves were collected to determine N content.

Number of nodes (i.e stolon) occurring along each primary branch were noted. Length of stolon was also measured on primary branches arising from mature rosettes. Inflorescences were measured for pedicel length and total number of flowers per mature rosette. Dry herb yields (DHY) per population obtained were after harvest.

Some of the germplasms of *C. asiatica* samples were used for transplantation. Mature seeds were collected from 17 different populations (Appendix 2) to determine seed characters.

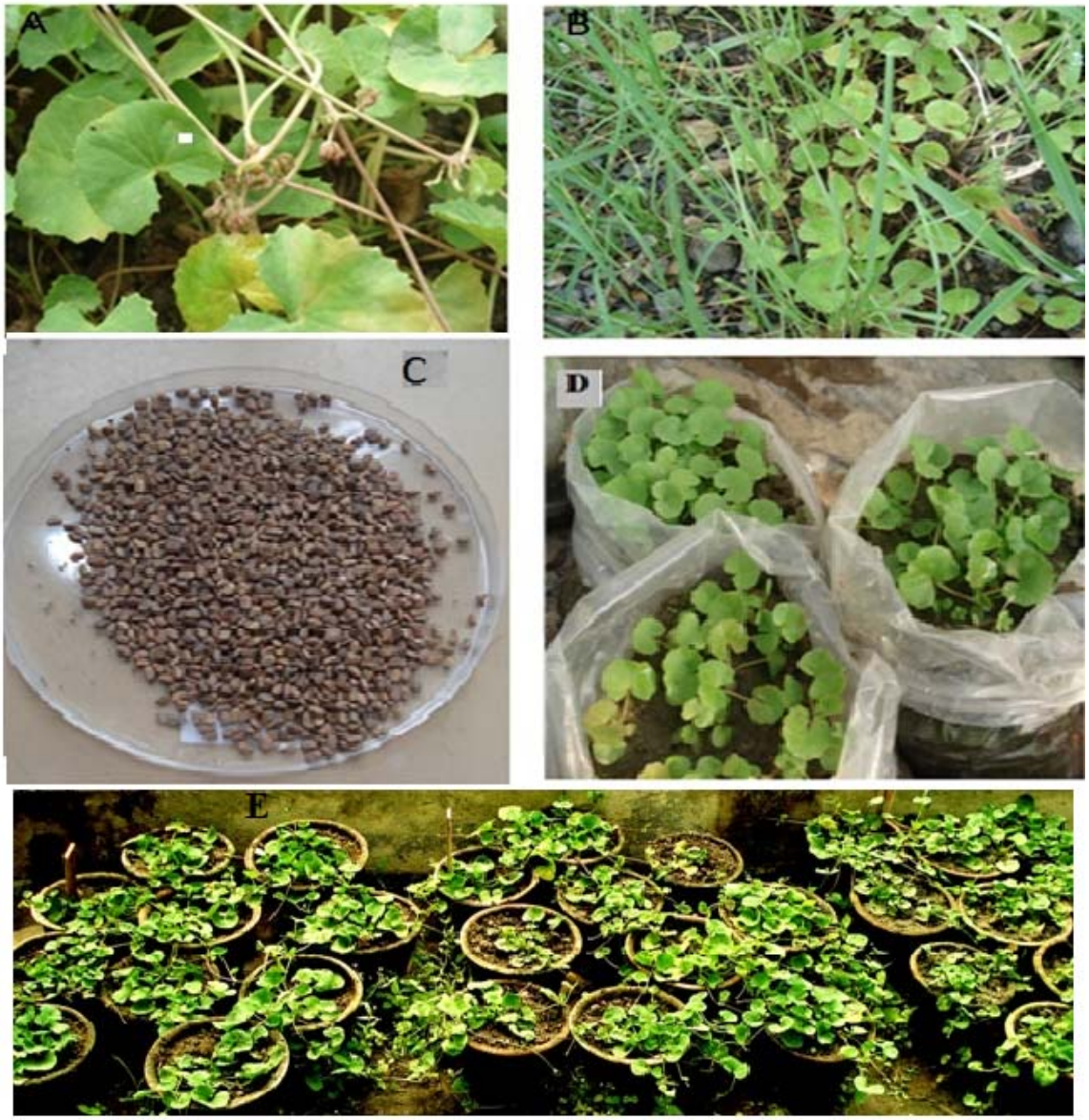


Plate 1. *Centella asiatica.* A) Flowering individual grown in earthern pot, B) Plants in natural habitat, C) Seeds, D) Seedlings, E. *Centella asiatica* grown in earthen pots in garden of Central Department of Botany, Kirtipur

4.2.3 Phenology

To record phenology, fifteen plots (10 m × 10 m) in three different habitats (shady grassland , open grassland, and open agricultural land) from Kirtipur and Matatirtha (five plots at each habitat) were subjectively chosen and marked. The plots were relatively undisturbed and had high density of *Centella asiatica*. In each plot, ten quadrats (1 m × 1 m) were located randomly. The number of individuals in each phenophase (i.e. seedling, vegetative, flowering and fruiting) was recorded in each quadrat. Twenty best looking ramets in each plot were marked for observing phenophase. Altogether, 150 quadrats lying within 15 plots were sampled. First sampling was done on February 15, 2008 and sampling was continued throughout the year in grassland and till July in open agricultural land (due to paddy plantation period). In repeated observations, the phenophases of marked individuals were recorded. Data of same phenophase from same habitat from two different locations were merged before analysis.

4.2.4 Collection of Plant Materials

Plant materials for phytochemical analysis and extraction of essential oil were collected from field. For phytochemical analyses, the aerial parts (leaves and stem) of *Centella asiatica* were collected from each of the 21 populations during May 2007 and May 2008. The collected plant materials were air dried in the shade for a week and sealed in zipper plastic till analysis. For extraction of essential oil, fresh mature plants of *C. asiatica* were collected in June 2008 from different three different habitats viz. open grassland, partially shaded grassland and shady grassland of Kathmandu valley and shade dried.

4.3 Transplantation

For analysis of genetic diversity and comparing phytochemicals of *Centella asiatica* between wild and transplanted, plantlets (30-40) from each of 21 populations were transplanted in October 2006 to Kathmandu (1350 m asl) in garden of Central Department of Botany, Tribhuvan University, Kirtipur and grown in earthen pots (mean diameter: 20 cm) filled with garden soil, sand and vermicompost (1:2:1) (Plate 1, E). Single individual was grown in each pot maintained under ambient environment and watered the plants as required.

Phytochemical constituents of transplanted plants were measured in May 2007. Fresh leaves collected from these plants were used for genetic study. Five samples from each population were used for genetic study.

4.3.1 Relative Growth Rate

In order to obtain the best estimate of the relative growth rate of *Centella asiatica*, the sixteen most similar stolon tip with respect to weight, number of nodes and the stage of development of the unexpanded leaf were then selected for use as experimental material from three different habitats of Kathmandu valley. The selected stolon tips were planted individually in 20 cm diameter earthen pots filled with soil, sand and vermicompost in ratio of 1:2:1. Initial fresh weights of plantlets were taken before transplantation. These pots were distributed randomly in a naturally lit glasshouse and and the plants were watered as required. The entire experiment was conducted during sunny days in late spring 2008.

At weekly intervals after planting, four replicates of each population were selected at random for harvesting (giving four harvests in all). These individuals were washed, bagged separately, labelled, and then oven dried at 70°C for 48 h. Dry weight of each individual was taken by electric balance (0.001 g). Relative growth rate (RGR) was calculated by using the following formulae (Zobel *et al.* 1987).

$$\text{RGR (g. g}^{-1}\text{ .mon}^{-1}) = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1}$$

Where, W_1 = total plant dry weight at time t_1, W_2 = total plant dry weight at time t_2.

4.4 Environmental Factors Affecting Growth

Experiments for the evaluation of different environmental conditions were performed on plantlets grown in garden of the Central Department of Botany (CDB), Tribhuvan University (TU), Kirtipur, Kathmandu, Nepal (85º17.32'E Long; 27º40.20'N Lat., 1350 masl). Plantlets were collected from most densely populated patch within a 5 m × 5 m area to reduce the probability of genetic differences among the plantlets. A pot culture experiment in a completely randomized design was established in the Botanical Garden of CDB, TU. All experiments were set in last week of October 2007 and the plants were harvested during May 2008. Plants were planted in earthen pot (mean diameter 20

cm). The different tested environmental conditions were: watering (125%, 100%, 70% and 30% field water capacity), light (70% shade, 50% shade and 30% shade), sand content in soil (0, 20, 40, 60, 80 and 100%) and integrated manuring of farmyard manure and urea in different proportion. Each treatment was repeated twice during succeeding season. Data were merged before analysis.

4.4.1 Effect of Moisture Level

The soil medium utilized for the experiment comprised clay (garden soil), horticultural sand and vermicompost in the ratio 1:2:1. Variation in different growth traits of *Centella asiatica* was investigated using a clone collected from Kirtipur site. The cuttings of plantlets were planted in earthen pots, one cutting per pot, which was filled with the soil as described above. The pots were then transferred to a glasshouse at CDB, TU Kirtipur; the temperature in glasshouse was higher (by 3 to 5°C) than outer environment. Planting was carried out in October 2007 and four different levels of water supply were applied [125% (surplus water), l00%, 70% and 30% of field capacity by weight]. Field capacity of soil was measured by following Zobel *et al.* (1987). Forty plants for each treatment were planted. A total of 160 plants were planted separately for the experiment. The first treatment (125%) was maintained by preventing drainage of excessive water from the pots by closing the hole lying at the bottom of the earthen pot. The pots were weighed every two days and additional water added to compensate the water loss by evapotranspiration. The soil moisture in the other treatments was maintained every two days at 100 %, 70 % and 30 % of field capacity. Fertilizer was not added during the experimental period. Weeding was done as required. All pots and treatments were rotated each week to counter any positional effects of pots within treatments. Some of the experimental plants were damaged by insects. As a result only thirty plants per treatment were randomly selected and were used for the observations. Data on yield and morphological traits were recorded in April 2008.

4.4.2 Effect of Soil Composition

The proportion of clay and sand varied by thoroughly mixing the clay soil (collected from agricultural land) with horticultural sand in different proportion. Six treatments (texture types) reflecting a gradient of decreasing clay content (%), 100 (S_1), 80 (S_2), 60 (S_3), 40 (S_4), 20 (S_5), and 0 (S_6) were prepared with increasing proportion of sand, the nitrogen and organic carbon content of test soil declined (Table 4.1).

The cuttings of plantlets were more or less of uniform size with four leaves. These were planted in earthen shallow pots filled with different types of soil (S_1 to S_6) in glass house. Altogether 300 plants, fifty plants for each treatment, were planted separately for experiment. Planting was done in October 2007 and equal amount of water was provided for irrigation to each treatment. All pots and treatments were rotated each week to counter any positional effects of pots within treatments.

Table 4.1 Soil textural classes and their characteristics

Soil type	Textural class	Bulk density (gcm^{-3})	Soil N (%)	Organic carbon (%)
S_1	Clay (0% sand)	1.55	0.25	4.5
S_2	Silt (20% sand)	1.40	0.22	4.2
S_3	Loam soil (40% sand)	1.35	0.19	3.8
S_4	Sandy loam (60% sand)	1.05	0.12	3.58
S_5	Sandy soil (80% sand)	0.99	0.09	1.31
S_6	Sand (100% sand)	0.88	0.008	0.025

4.4.3 Effect of Light Level

The cuttings of plantlets were nearly uniform in size with four leaves; they were planted in earthen pots filled with a mixture of field soil, sand and vermicompost (1:2:1) in glass house. Altogether 160 plants, forty plants for each treatment, were planted separately for the experiment. Planting was done in October 2007. After two weeks they were then transferred to three distinct shading levels (30, 50 and 70%) and full sunlight as control. The light was controlled by various layers of nylon-net placed 2 m above the ground. Light intensity was measured by lux meter (LX-101, Taiwan).The average light intensity measured was T_1 (1125 $\mu mol \cdot m^{-2} \cdot s^{-1}$), T_2 (645.82 $\mu mol \cdot m^{-2} \cdot s^{-1}$), T_3 (402.38 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and T_4(296.96 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The plantlets were irrigated at regular interval depending on the weather and soil moisture status.

4.4.4 Effect of Integrated Manuring

Total amount of nitrogen used was 20 kg/ha as recommended dose by National Agricultural Research Council for vegetables in Kathmandu valley (Anonymous 2006). The recommended dose of N was fulfilled from two different sources: FYM (cattle manure) and urea mixing in different proportions with soil. The soil used for the experiment was clay-loam, collected from garden of Central Department of Botany. FYM used for study was 5-8 month old, prepared for crop field. Five replicas of both soil samples and FYM were tested for nutrient content. The protocol used for soil nutrient analysis was the same as used for soil analysis of habitat of *C. asiatica*. The chemical properties of the soil substrate and FYM have been shown in Table 4.2. Six treatments reflecting a gradient of decreasing FYM content (%), 100:0 (T_1), 75: 25 (T_2), 50: 50 (T_3), 25:75 (T_4), 0:100% (T_5) and control soil without manure (T_6) were prepared (Table 4.3).

Table 4.2 The chemical properties of the soil substrate and FYM manure

Attributes	pH	N (%)	OC (%)	OM (%)
FYM (Cattle manure)	8.2±.12	1.21±.08	20.12±1.3	34.64±1.2
Soil	5.83±.08	0.14±0.02	1.32±0.90	13.14±0.12

The cuttings of plantlets were more or less of uniform size with four leaves in each. Single plantlet was planted in every pot (average diam. 20 cm). The pots were irrigated whenever necessary. Altogether 240 plants, forty plants for each treatment, were planted separately for experiment. Planting was done in October 2007. All pots and treatments were rotated each week to counter any positional effects of pots within treatments.

Table 4.3 Treatment conditions

Treatments	T_1(100:0)	T_2(75:25)	T_3(50:50)	T_4(25:75)	T_5(0:100)	T_6
(Urea :FYM) (g)	0.6:0	0.4:5.0	0.3:10.0	0.15:15.0	0:20.0	Control (No manure)

4.4.5 Growth Measurement

Data on yield and morphological traits were recorded in April 2008 and 2009. Thirty to forty plants per treatment, selected randomly, were used for the observations. Ninety mature leaves per treatment were measured for leaf length (LL), leaf width (LW), petiole length (PL), leaf area (LA), dry weight of leaf (LDW) and specific leaf area (SLA). Length and width of leaves, and petiole length were measured in fresh leaves. Then these leaves were oven dried (60ºC, 48 h) and mass of each leaf was weighed in electric balance (0.001 g). Length and width of leaves were measured and multiplied by conversion factor following Zobel *et al.* (1987) to determine leaf area. SLA was calculated as the ratio of leaf area and dry mass. Twenty samples of leaf were collected from each treatment to determine leaf N content.

Number of nodes (NND) occurring along each primary branch were noted. Internodal lengths (IND) were also measured on primary branches arising from mature rosettes. The number of leaves (NLN) and primary branches (NBN) arising from it were also scored. Inflorescences were measured for flower pedicel length (FPL) and total number of flowers per mature rosette. Fresh (FHY) and dry (DHY) herb yields per treatment were obtained after harvest and moisture content (MC) calculated. Chlorophyll a, chlorophyll b and total chlorophyll content was determined following the method of Arnon (1949) in five samples from each treatment.

4.5 Laboratory Analysis

4.5.1 Soil and Leaf Material Analysis

Soil samples were air dried in shade under laboratory condition and the dried soil was passed through the fine sieve (0.5 mm). Soil organic matter (OM) (Walkley and Black method) and the total nitrogen (N) (micro-Kjeldahl method) were determined following the methods described by Gupta (2000). Altogether 630 soil samples from the habitats of *Centella asiatica* were analyzed. Leaf N content was determined by modified micro-Kjeldahl method following the procedure described by Horneck and Miller (1998). One thousand six hundreds and sixty leaf samples of *Centella asiatica* (six hundred and thirty samples each from wild and transplanted and four hundreds from pot experimental plants) were analyzed for nitrogen concentration. The analysis was carried out in the laboratory of Central Department of Botany, Tribhuvan University.

The laboratory protocol used for analysis of soil and leaf has been described in section 4.5.1.1**.**

4.5.1.1. Protocol for Soil Analysis

Soil pH

Soil pH was determined by the potentiometric method (Gupta 2000) using a pH meter (Digital pH meter,802, systronics (89-92) Naroda Industrial Area, Ahmeda Bad, India). Before pH measurement, the electrode of the pH meter was dipped for 24 h in tap water. Then, buffer solutions of pH tablet 7.0 and 4.0 were prepared freshly. The pH meter was warmed up for 15 min. before starting pH measurement. 10g of air dried fine soil was mixed in 100ml of distilled water and stirred well by the help of glass rod. Then, the mixture of soil and water was left for decantation about half an hour and hence solution of soil sample was made ready for pH measurement. Now, the pH meter was callibrated through buffer solution of pH 4.0 and 7.0 and pH measurement was taken for each solution of soil sample. Electrode of pH meter was flushed by distilled water and wiped by cotton each and every time before dipping it from anyone solution either buffer or of soil sample to next.

Organic Carbon and Organic Matter

Dried and fine (passed through 0.5 mm sieve) soil (0.5g) was taken in a conical flask (500 ml) and added 10 ml potassium dichromate (1N) with gentle swirling. Concentrated sulphuric acid (20 ml) was added and the mixture was allowed to cool down. After 30 minutes, 200 ml of distilled water was added to the mixture. After adding 10 ml phosphoric acid and 1ml diphenyl amine indicator, the mixture was titrated with freshly prepared ferrous ammonium sulphate (0.5N) until the color changed from blue-violet to green. In every bath of 10 soil samples, a single blank (without soil) was run. Organic carbon and organic matter was calculated as follows:

$$\text{Organic Carbon (\%)} = \frac{1.3 \times 0.003 \times 100 \times N(B-C)}{M}$$

Where, N = Normality of Ferrous ammonium sulphate

B = Volume of Ferrous ammonium sulphate consumed in blank titration (ml)

C = Volume of Ferrous ammonium sulphate consumed with soil sample (ml)

M = mass of soil (g)

Organic matter (%) was obtained by multiplying organic carbon content by Van Bemmelen factor (1.724).

Nitrogen: Estimation of total nitrogen by micro- Kjeldahl method included three steps: digestion, distillation and titration. In digestion, 1g air dried and sieved (passed through 0.5 mm sieve) soil was taken in a clean and dry kjeldahl digestion flask (300 ml). As a catalyst, mixture of 3.5 g potassium sulphate and 0.4 g copper sulphate was mixed with soil sample, and 6 ml concentrated sulphuric acid was added to the mixture with gentle shaking. The flask was heated at low temperature until the bubbles disappeared from the black mixture. Then the temperature was raised to about 350°C and heating was continued until the mixture turned to turquoise (greenish – blue). The flask was removed from the heating mantle and allowed to cool down for about 15 minutes. Then 50 ml distilled water was added to the digest with shaking. For distillation, the digest mixture was transferred to distillation flask (300 ml). Boric acid indicator (10 ml) was taken in a small beaker (100 ml) and placed below the noggle of condenser in such a way that the noggle was dipped into the indicator solution. When the mixture in the distillation flask was slightly warm, 30 ml of sodium hydroxide solution (40%) was added. When distillate began to accumulate in the beaker with boric acid indicator, color of the indicator changed from pink to green. Distillation was continued until the volume of distillate reached to about 50 ml. Then the distillate was titrated with hydrochloric acid (0.1 N) and the volume of acid consumed was recorded. With each batch of soil samples, single blank (without soil) was run. Following formula was used to determine total nitrogen content of the soil samples:

$$\text{Soil N (\%)} = \frac{14 \times N \times (S-B) \times 100}{M}$$

Where, N=Normality of HCl

S= Volume of HCl consumed with sample (ml)

B=volume of HCl consumed with blank (ml)

M= mass of soil taken (mg)

Leaf Nitrogen (N) Content

Leaf N content was determined by modified micro-Kjeldahl method following the procedure described hy Horneck and Miller (1998). Estimation of leaf N content also included three steps: digestion, distillation and titration. Oven dried (60°C for 72 h) leaf sample (250 mg) was taken in a clean and dry kjeldahl digestion flask (300 ml), and 2 g catalyst(mixture of potassium sulphate, copper sulphate and selenium powder in the ratio of 100:10:1 was added to it. After adding 6 ml of concentrated sulphuric acid to the mixture of leaves and catalyst, the flask was heated at low temperature until the bubbles ceased to ooze out. Then temperature was raised to about 350°C and heating was continued until the color changed from black to yellowish and finally to turquoise. Once the final color appeared the flask was immediately removed from the heating mantle and allowed to cool down. After about 15 minutes, 50 ml of distilled water was added gradually to the mixture. With each batch of leaf samples, single blank (without leaf) was run. Further steps of distillation and titration were same to N estimation in soil.

4.5.2 Seed Germination

4.5.2.1 Seed source and physical attributes

Seeds of *Centella asiatica* were collected from 17 different populations (Table 3.1) during June 2006 and June 2007. Seeds were dried in shade for a week and stored in hermetically sealed polythene pouches (Copeland 1976) under ordinary storage condition at room temperature for subsequent germination studies. Seed attributes of all 17 populations were measured. Seed output per unit area was measured according to Zobel *et al.* (1987). The seed size was measured using measuring scale, whereas seed mass was determined using an electric balance (0.001 g). Due to insufficient amount of seeds collected from other locations, germination experiments were performed only for seeds collected from Kirtipur.

4.5.2.2 Seed viability

The seed covering was removed manually and the decoated seeds of the plant species were soaked in distilled water and kept in dark for 24 h before being treated with 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for assessing the effects of various durations of storage on viability (Misra 1968).

4.5.2.3 Germination test

Germination of *Centella asiatica* was evaluated between August to October (2-3 months old seeds) by placing seeds in petri dishes (diameter 9 cm) containing two layers of Whatman No. 1 filter paper, moistened with 10 ml of distilled water or a treatment solution. The petri dishes were placed on a table in the laboratory at room temperatures. The average daily maximum and minimum room temperatures during the experiment were 30 and 20ºC, respectively. The petri dishes were wrapped in two layers of aluminium foil to determine germination in complete darkness. Seed germination was monitored 2 days after the start of the experiment at the interval of one week with the criterion for germination being visible protrusion of the radical till six weeks. All sets of treatments and control contained four replicates, each of 30 seeds. Germination experiment was carried out in the laboratory of Central Department of Botany, Tribhuvan University.

4.5.2.4 Effect of pre-treatments

Presoaking treatments used in the study were: different concentration of GA_3 treatment, warm water, cold water and 10% HNO_3. Before placing in petri dishes, seeds were soaked in 10, 20, 30, 40, 50 and 100 ppm GA_3 solution; 40 and 60ºC warm water and cold water (at room temperature) for 30 min. For 10% HNO_3 treatment, after soaking the seeds in the solution, washed with distilled water three times before incubation in Petri dishes.

4.5.2.5 Effect of light

To study the effects of coloured light, petri dishes with seeds were covered by red and blue cellophane plastic till the completion of experiment.

4.5.2.6 Effect of Salt stress

Seeds of *Centella asiatica* were incubated in sodium chloride (NaCl) solutions of 0, 500, 1500, 2500, 3500, 4500, 6500 ppm to determine the influence of salt stress on germination. The non germinated seeds at 6500 ppm were transferred to petri dishes containing 5 ml of distilled water and placed in the incubator as described previously. The seeds were rinsed before being transferred.

4.5.2.7 Phytotoxic effect of leaf leachate of alien plants

To study the effects of leaf leachate, four alien plant species *viz*. *Chromolaena odorata, Parthenium hysterophorus, Ageratum conyzoides* and *Xanthium strumarium* were collected randomly in vegetative stage in a sunny mid day of July 2007 from Kirtipur. Leachates were prepared by immersing 10 g plant materials (leaf, stem) of each species in 100 ml of distilled water in beaker separately and kept for 24 h at room temperature and filtered using muslin cloth as recommended by Tukey and Mecklenburg (1964). This filtrate was taken as stock solution with 10% of concentration and the solution of different concentrations (i.e. 0%, 5%) were prepared by dilution with distilled water and used for petri dish bioassay against test seeds with the same procedure as stated above.

4.5.3 Phytochemical Analysis

Aerial parts of *Centella asiatica* collected from each site were shade dried and used for phytochemical analysis. After sample collection, further phytochemical analysis work was carried out in Laboratory of Pharmaceutical Sciences, Padova University, Italy.

4.5.3.1 Extraction of plant samples

Dried aerial parts of plant were ground and 100 mg were placed in a 15 ml-falcon tube (screw-capped polypropylene centrifuge tube) and extracted three times with 5.0 ml of methanol by sonication with Falc Ultrasonic UT460 bath (50 KHz). The extraction was performed at room temperature. The extract was centrifuged for 5 min at 3000 rpm and the supernatant was combined in a 50 ml volumetric flask, diluted to final volume with methanol and mixed thoroughly. All samples were filtered through a 0.45 μm PTFE syringe filter prior to injection in HPLC.

4.5.3.2 HPLC condition

Instrument consisted of an Agilent 1100 series liquid chromatograph equipped with Agilent 1100 Diode Array (DAD) and SEDEX LT60 Evaporative Light Scattering Detectors (ELSD). An Agilent XDB-C-18 reverse phase column (25 × 4.6 mm, 4.6 μm) was used as stationary phase. The gradient elution program, with aqueous formic acid (0.1%) (A) and acetonitrile (B), was: 0-8.5 min, linear gradient from 12 to 26% B; 8.5-11 min, isocratic conditions at 26% B; 11-16 min, linear gradient from 26 to 40% B; 16-45 min, linear gradient from 40 to 50 % B; 45-50 min, linear gradient from 50 to

100% B. Flow rate was 1 ml/min and injection volume 20 µl. The ELSD detector temperature was 50°C, nitrogen pressure 2.2 bars, and the gain level 10 (a.u.).

Calibration curves were obtained by preparing standard solutions, as listed in Table 4.4. Asiaticoside and asiatic acid were determined with the ELSD detector. Chlorogenic, chicoric and rosmarinic acids were determined with the DAD at 330 nm, and at 350 nm for kaempferol, quercetin and quercetin 3-*O*-glucuronide. HPLC chromatogram of the standard compounds is reported in Figure 4.1.

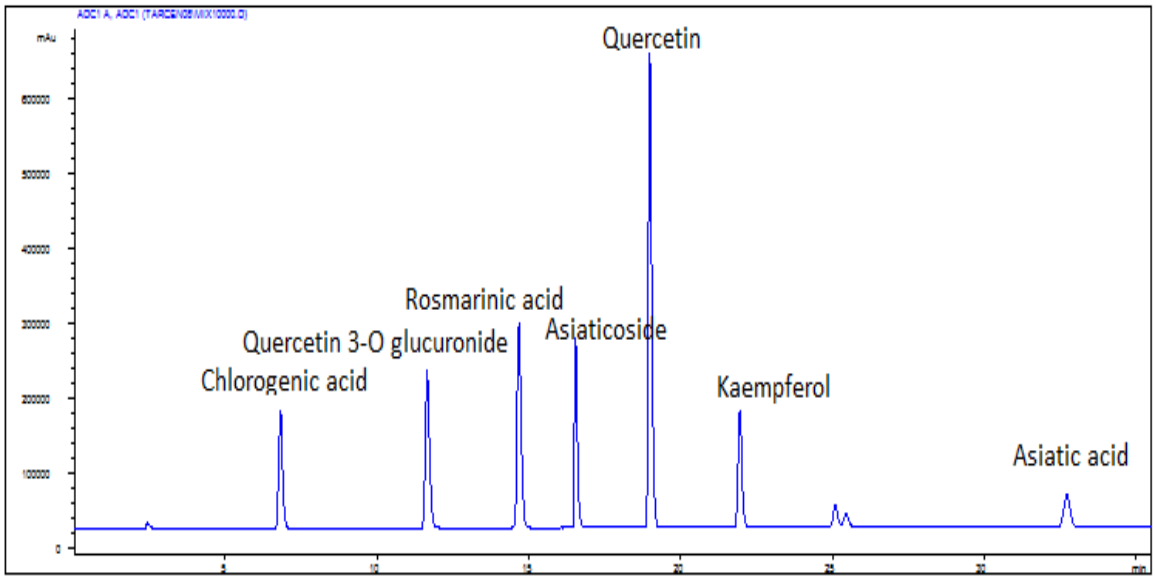


Fig 4.1 HPLC chromatogram of the standards used for the quantitative determination of chemical components.

4.5.4 Essential Oil

4.5.4.1 Extraction of the essential oil

Two hundred gram of shade dried plant from three different habitats (i.e. partially shaded grassland, open grassland and open agricultural land) from Kathmandu valley were collected and hydrodistilled with a Clevenger apparatus for 4 h. Pale yellow oil was collected and stored at 4°C in the dark for analysis.

Table 4.4 Concentration ranges and calibration curves for the analyzed secondary metabolites.

Analyte	Concentration (μg/mL)	Regression curve[a]	R^2 (n=6)	LOD (μg/mL)	LOQ (μg/mL)
Asiatic acid	4.25-100.0	Log y = 0.608 Log x-1.880	0.998	1.04	3.46
Asiaticoside	5.62-140.5	Log y = 0.588 Log x-1.872	0.999	1.02	3.40
Chicoric acid	0.732-73.17	y =0.0127x-0.0886	0.999	0.29	0.97
Chlorogenic acid	0.685-97.53	y=0.0168x-0.2456	0.999	0.40	1.33
Rosmarinic acid	0.766-113.0	y=0.0147x+0.1534	0.999	0.35	1.17
Quercetin	0.84-84.03	y=0.0086x+0.9724	0.998	0.21	0.70
Quercetin -3-O-glucuronide	1.3-130.0	y=0.0393x-0.0249	0.998	0.10	0.33
Kaempferol	0.66-65.97	y=0.0136x+0.391	0.999	0.19	0.63

[a] x= peak area; y concentration of analyte (μg/mL).

4.5.4.2 GC-MS analysis

Essential oil of *Centella asiatica* was analyzed by gas chromatography coupled with mass spectrometry (GC-MS) (Varian Saturn 2000 GC-MS series, using a fused-silica-capillary column, DB-5, 30 m × 0.25 mm × 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). GC-MS analysis was obtained using the following conditions: carrier gas He; flow rate 0.8 ml/min; split 1:10; injection volume 1μl; injection temperature 250°C; oven temperature 45°C for 5 min and progress from 45°C to 220°C at 3°C/min, from 220°C to 250°C at 10°C/min and holding at 250°C for 5 min. The ionization mode used was electronic impact at 70 eV. Identification of the components was achieved by comparison of their mass spectral fragmentation patterns with those stored in the data bank (Wiley library) and by their retention indices.

GC-FID analyses were carried out on Agilent 6840 N gas chromatograph equipped with a FID detector. The analytical conditions were the same as described for GC-MS analysis. The FID temperature was at 220°C. The percentage composition of the oil

was computed by the normalization method from the GC peak areas, without using correction factors.

4.5.5 Genetic Diversity

4.5.5.1 Morphological analysis

Thirty individuals from each population from transplanted samples were taken for morphological analysis. All other morphological traits were measured two times in successive growing season as in wild samples (described in Section 4.2.2).

Pigment analysis: Chlorophyll a, Chlorophyll b and total chlorophyll content was determined following the method of Arnon (1949) in five samples from each population.

4.5.5.2 Molecular analaysis

Molecular work was carried out in the Laboratory of Biotechnology Unit of Nepal Agricultural Research Council, Khumaltar, Lalitpur.

Isolation of genomic DNA: Fresh leaves from transplanted samples were used for genetic study. Five to seven samples from each population were used for study. DNA was isolated by using CTAB (Cetyl Trimethyl Ammonium Bromide) (Sigma Aldrich, USA) protocol developed by Doyle and Doyle (1987) with slight modification. Young leaf tissue (0.3 g) ground into fine powder in liquid nitrogen and transferred to 600 µl of preheated (65°C) extraction buffer [2% CTAB]. The slurry was incubated for 10 minutes in a 65°C water bath. An equal volume of chloroform: isoamylalcohol (24:1 v/v) (Qualigens Fine Chemicals) was added to the extract prior to centrifugation at 14,500 rpm for 26 sec at room temperature. To the supernatant again an equal volume of chloroform: isoamylalcohol (24:1 v/v) (Qualigens Fine Chemicals) was added and centrifuged at 14,500 rpm for 26 sec at room temperature The DNA was precipitated by adding ice cold absolute ethanol (Sigma Aldrich) and centrifuged at 8000 rpm for 1 min at room temperature. The pellet was washed with 70% ethanol (Sigma Aldrich). The pellet was dissolved in 50µl TE (Tris-EDTA) buffer and stored at –20°C. DNA quality and quantity were evaluated spectrophotometrically at 260/280 nm and the DNA concentrations were rechecked by visual assessment of band intensities on 0.8% agarose gel in comparison to Lambda DNA marker. DNA samples were diluted with sterile Milli Q water to 50 ng/µl for further use in RAPDs.

Random Amplified Polymorphic DNAs (RAPD) analysis: In a preliminary study, 21 decamer primers of arbitrary sequence (Kits A and C provided by Operon Technologies Inc., Alameda, CA, USA) were tested for PCR amplification. Out of which, only 8 primers produced scorable and reproducible bands, so these primers were selected for further experiments. DNA amplification was carried out in PCR plates with a total reaction volume of 25 µl. The reaction mixture contained 12.5 µl of Go Taq Green Master mix obtained from Promega Corporation,USA (Taq DNA polymerase, dNTPs, $MgCl_2$, reaction buffer and dye), 1 µl primer(0.5µM), 7.5 µl de-ionised water and 4 µl DNA(25 ng). DNA amplification was performed using a DNA Thermocycler (MJ Research Inc., USA). Each tube was added with 10 µl of sterile mineral oil to seal the reaction mixture and to prevent evaporation. The thermocycler was programmed for an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 45 s at 94°C, 1 min at 37°C, extension was carried out at 72°C for 1 min and final extension at 72°C for 7 min.

After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1×TAE buffer. Agarose gel was stained with ethidium bromide and photographed under UV light (EASY Win32, Herolab, Germany). Electrophoresis was performed at a constant voltage at 60 V for 3 h. The amplicons were visualized under UV light and photographed. The gel was also documented by Gel Doc 2000 (Bio-Rad, USA) for scoring the bands. The amplicon size was determined by comparison with the ladder (Gene Ruler 100 bp ladder plus; Sigma Chemical Company, St. Louis). The entire process was repeated at least twice to ensure reproducibility.

For data analysis, a binary matrix reflecting specific RAPD band presence (1) or absence (0) was generated. Similarity matrix among individual plants in the study was estimated according to the Jaccard's formula. The dendrogram was constructed based on similarity matrix by Jaccard's index (Jaccard 1901) method with arithmetical average (UPGMA) using cluster analysis.

4.6 Numerical Analysis

Before comparison of mean of ramet density, aboveground biomass, morphological traits, and reproductive output among sampling sites, the data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test). Mean across

the sites were compared by analysis of variance (ANOVA), and the pairs of sites were compared by multiple comparison using Duncan Homogeneity test (variance assumed equal). Leaf N content met the assumptions of normality and homogeneity of variance; therefore ANOVA, followed by Duncan multiple range test was used for comparison of mean across sites. All growth traits data of pot experiment were also compared by ANOVA, followed by Duncan multiple range test. All statistical analyses were done using Statistical Package for Social Sciences (SPSS 2002, Version11.5).

For genetic diversity study, a dendrogram of morphological traits was constructed using software SPSS (11.5) by hierarchical clustering method. The binary data matrix, reflecting specific RAPD band presence (1) or absence (0), was analyzed to assess the similarity coefficient among studied populations following Jaccard's formula (Jaccard 1901). To illustrate relatedness among populations, the presence-absence matrix of RAPD bands was analyzed based on Jaccard's distance and unweighed pair group method with arithmetical average (UPGMA) in NTSYS pc programme version 2.1 (Rohlf 2000) .

5. RESULTS

5.1 Distribution

Centella asiatica was collected from throughout Nepal (eastern, central and western) from < 85 to 2300 m asl in a wide range of habitats from open moist fallow land, forest gap to roadside to shady grassland (Table 3.1, Fig 5.1). While analyzing total 70 specimens collected from Nepal; 50% specimens were from open moist places, 38.11% specimens from partially shaded grassland, 10.52% from agricultural fallow land and only 1.31% specimens were collected from forest gap (Fig 5.1 ,A). Regarding climatic zone, 50% specimens were collected from tropical belt, 38% specimens were collected from sub tropical belt and only 12% specimens were collected from temperate belt (Fig 5.1 B).

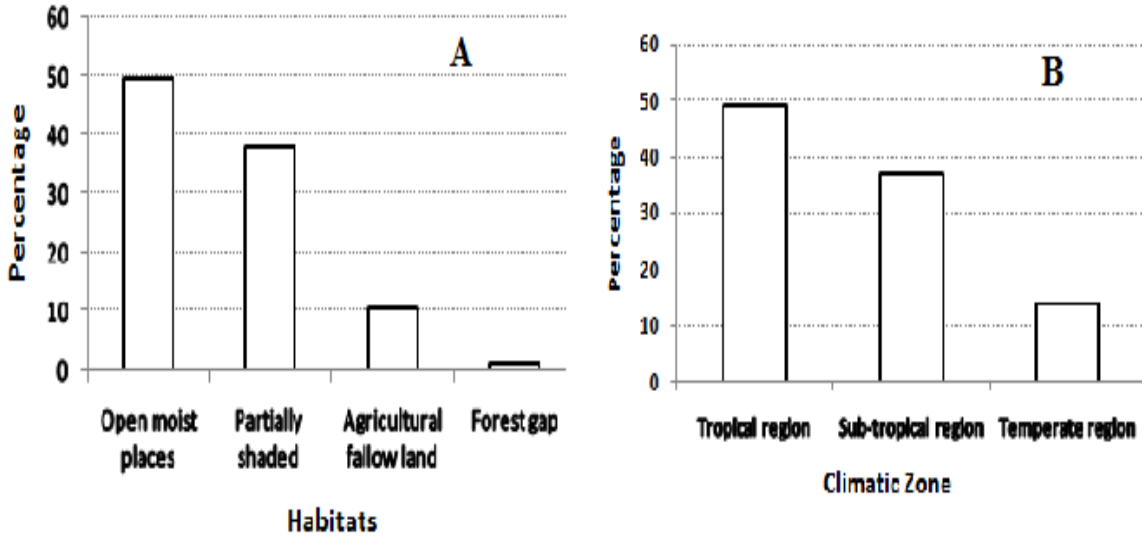


Fig. 5.1. Specimens of *Centella asiatica* in National Herbarium (KATH)
A.different habitats , B.climatic zone

5.2 Abundance

Population density and above ground biomass yield varied with habitats (Table 5.1). Mean values of plant density and biomass across the habitats were 72.53 pl/m^2 and 37.95 g/m^2, respectively. Highest density was shared by relatively undisturbed shaded grassland. Partially shaded grassland had highest ramet density (103.24 plants/m^2) and biomass (54.5 g/m^2) yield. Open agricultural land had least density (33.12 pl/$m^{2)}$ and biomass (20.12 g/m^2) yield. Density and biomass production of *C. asiatica* were high in areas rich in soil nitrogen and organic carbon (Table 5.1 & 5.15).

Regarding ecological region, plant density ranged from 75.91 (western region) to 122.32 plants/m^2 (eastern region) (Fig 5.2). Plant biomass was also significantly higher (61.6g/m^2) in eastern Nepal. Although density and plant biomass of *Centella asiatica* were not correlated (r = 0.182 & 0.012, respectively) with altitude of collection (Fig 5.3) & plant bimass of lower altitude seems relatively higher than higher altitude (Fig 5.4).

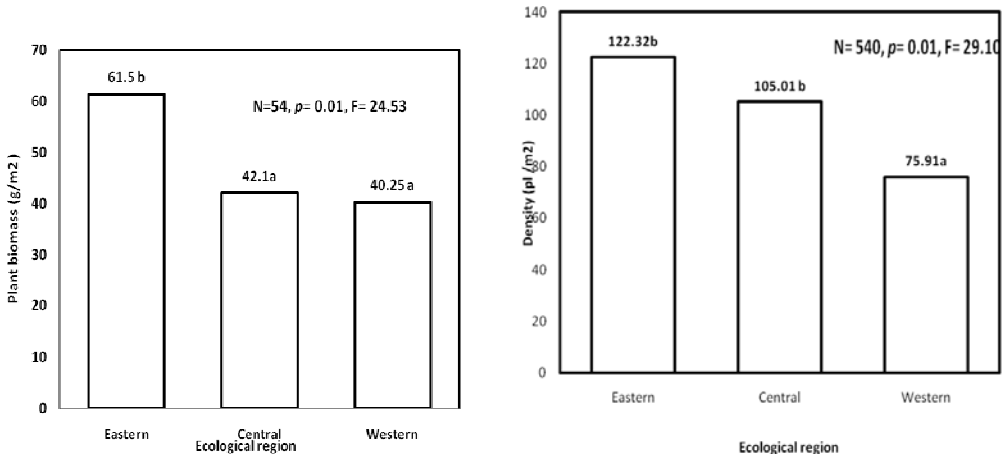


Fig 5.2 Density and plant biomass (g/m^2) of *Centella asiatica* at different ecological region of Nepal.Main entries above bars are means;values with same letter are not differed significantly.

Table 5.1 Density and morphological characters of *Centella asiatica* in different habitats in Nepal. For each parameter significant difference between mean among different sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes	Partially shaded grassland	Open Grassland	Open agricultural Land	N	Mean	F value	P value
Density (plants /m^2)	103.24^{c} ±2.09	81.14^{b} ±1.43	33.12^{a} ±1.57	540	72.53±1.69	22.21	< 0.001
Petiole length(cm)	9.41^{c} ±0.25	5.91^{b} ±0.4	2.36^{a} ±0.89	1890	5.89 ±1.02	47.95	< 0.001
Leaf length(cm)	2.66^{b} ±0.53	1.23^{a} ±0.96	1.22^{a} ±0.34	1890	1.78 ±0.71	40.21	< 0.001
Leaf width (cm)	4.43^{c} ±0.90	2.69^{b} ±0.80	1.92^{a} ±0.60	1890	3.65 ± 0.77	4.48	<0.001
Stolon length (cm)	7.28^{c} ±1.29	5.88^{b} ±0.98	3.15^{a} ±1.08	540	5.90 ± 2.19	3.59	0.076
SLA cm^2/g	498^{c} ±41	342^{b} ±26	189^{a} ±29	1890	343 ±33	9.74	< 0.001
Leaf no. /ramet	3.26^{a} ±0.89	3.57^{a} ±1.08	3.64^{a} ±0.82	540	3.49±0.96	0.254	0.857
Plant biomass (g /m^2)	54.5^{c} ±0.89	39.23^{b} ±1.20	20.12^{a} ±0.28	540	37.95 ±0.79	18.50	< 0.001
Flowers no./ramet	3.24^{a} ±1.14	6.68^{b} ±2.53	10.56^{c} ±2.36	540	6.82 ±2.01	33.35	< 0.001
Seeds/ ramet	0.24^{a} ±0.03	2.16^{b}±1.21	9.65^{c} ±0.62	540	4.08±0.23	28.14	< 0.001

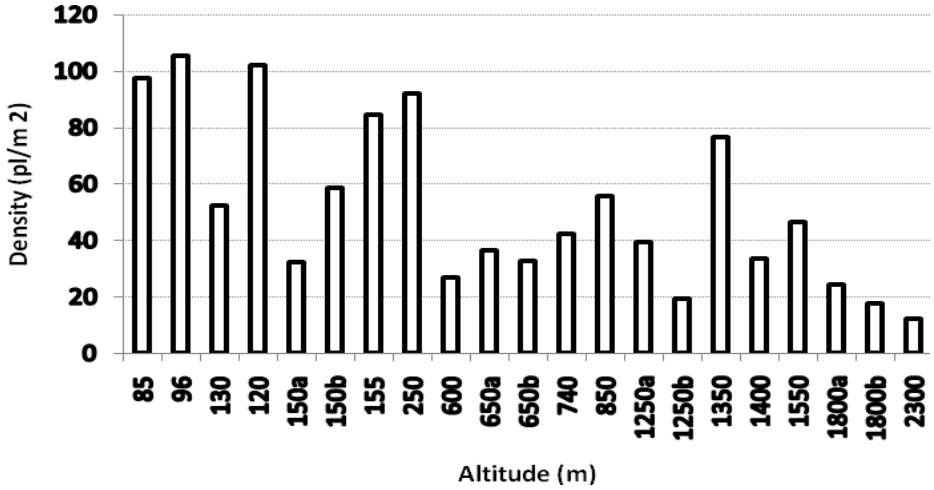


Fig 5.3 Density (pl/m^2) (N=540) of *Centella asiatica* at different altitudinal range.

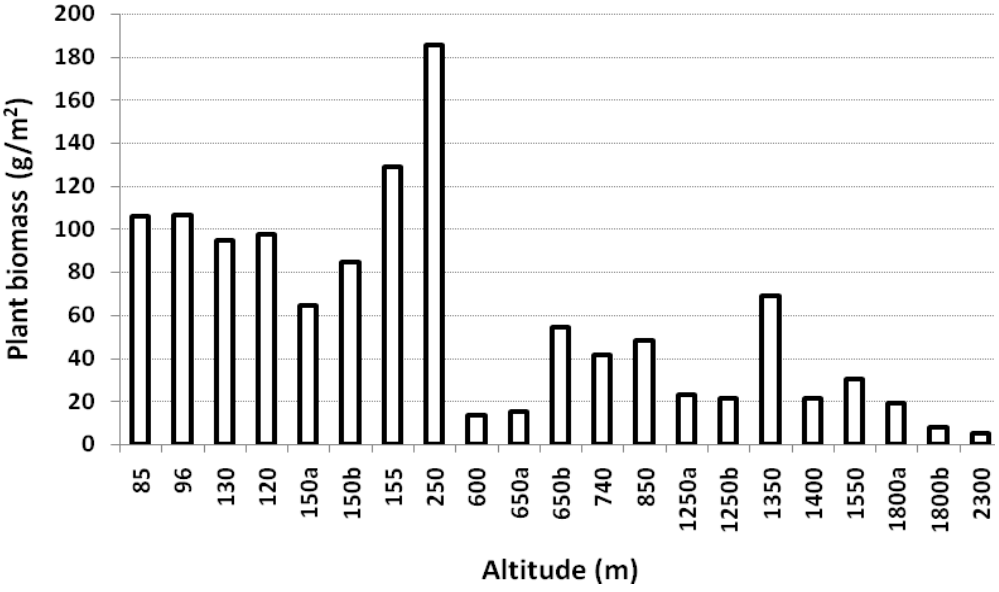


Fig 5.4 Biomass production (g/m^2) (N= 54) of *Centella asiatica* at different altitudinal range.

5.3 Leaf Traits

Leaves were annual, which remained alive for 6-8 months from early summer to early winter. Leaves were reniform in shape, and light green to dark green in colour. Petiole length ranged from 2.36 to 9.40 cm with mean value 5.89 cm (Table 5.2). Length of individual leaf was measured upto 2.66 cm (mean: 1.78 cm, N = 1890). The flower was pinkish-purple. Flowering was asynchronized. Average number of flowers per ramet

was 6.82 (N = 540). Fruit was pumpkin shaped. A single plant produced an average of 14.11 seeds (N = 1700) (Table 5.4).

Table 5.2 Growth traits and biomass production of *Centella asiatica* for entire range of sampling sites. SD: Standard deviation, CV: Coefficient of variation

Attributes	Sample size	Minimum	Maximun	Mean ± SD	CV
Petiole length (cm)	1890	2.36	9.40	5.89 ± 1.92	32.59
Leaf length (cm)	1890	1.22	2.66	1.78±0.52	29.21
Leaf width(cm)	1890	1.90	4.41	3.65 ± 0.78	21.36
Specific leaf Area(cm^2/g)	1890	189	498	343 ± 229	66.76
Leaf Nitrogen (%)	630	0.67	3.02	1.76 ± 0.44	25.16
Stolon length(cm)	540	3.15	7.28	5.43±2.45	45.42
Leaf number/ramet	540	2.56	5.03	3.83± 0.75	19.58
Flower number	540	3.42	10.56	6.82± 2.58	37.82
Dry mass of plant (g)	540	0.24	2.19	0.98± 0.5	51.02

5.4 Variation in Growth and Reproduction

There was a significant difference in growth (petiole length, specific leaf area, leaf mass and stolon length) and reproductive traits (flowers/plant, seeds/plant) of *Centella asiatica* in different habitats. Petiole length ranged from 2.36 cm at open agricultural land to 9.41 cm at partially shaded grassland (Table 5.1). There was significant difference (p = <0.001) in leaf length and leaf width of individual leaf among the habitat. Specific leaf area ranged from 189 to 498 cm^2/g (mean 343 cm^2/g, N = 1890). Average number of leaves per ramet was not affected by habitats. The number of flowers per ramet was highest (10.56) in open agricultural fallow land. The number of flowers per inflorescence in open agricultural land was three times and two times higher than in partially shaded grassland and open grassland, respectively.

Centella asiatica was found extensively from eastern to western Nepal. There was significant difference in growth traits among individuals of different ecozones (Figs 5.5-5.6). Petiole length, leaf length and leaf width were significantly higher in samples from eastern Nepal than from other regions (Fig 5.5) Specific leaf area (SLA) ranged

from 287.46 (eastern region) to 425.00 cm^2/g (central region) with mean value 347.08 cm^2/g (Fig 5.6). The number of flowers per ramet also differed significantly (p = 0.009).

There were significant positive correlation between SLA and leaf length and width (Table 5.3). Dry mass of individual plant was positively correlated with all measured growth traits but negatively correlated with asiaticoside (r = –0.103) and asiatic acid (r = –0.10) content of plant and the relation was not significant with other chemical constituents.

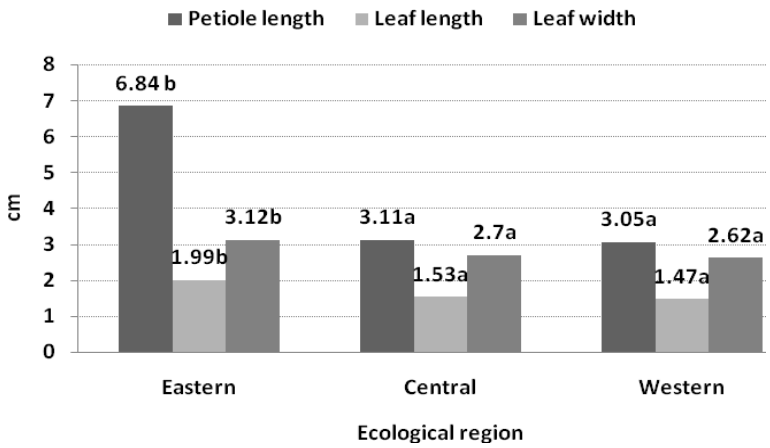


Fig 5.5 Leaf characters of *Centella asiatica* at different ecological regions of Nepal. Main entries above bars are means;values with same letter are not differed significantly.

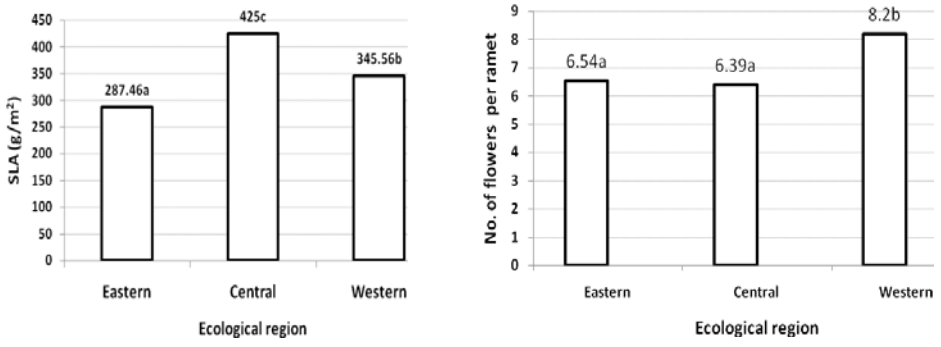


Fig 5.6 Specific Leaf Area(SLA) (N=1890) and number of flowers per ramet (N=540) of *Centella asiatica* at different ecological regions of Nepal. Main entries above bars are means ;values with same letter are not differed significantly.

Table 5.3 Correlations coefficients of growth traits and yield components of *Centella asiatica.*

Attributes	Petiole length	Leaf length	Leaf width	SLA	Leaf N	Stolon length	Flowers /ramet	Dry mass/ plant	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O glucuronide	Kaempferol
Leaf Length	0.29**														
Leaf width	0.26**	0.66**													
SLA	ns	0.08*	0.14**												
Leaf nitrogen	ns	ns	0.21**	0.15**											
Stolon length	0.33*	0.26*	0.08*	ns	ns										
Flowers/ramet	ns	ns	ns	ns	ns	.11**									
Dry mass/plant	0.24**	0.18**	0.07**	0.18**	0.15**	0.31**	0.12**								
Asiaticoside	ns	ns	ns	-0.21*	ns	ns	0.34	-.10*							
Asiatic acid	ns	ns	ns	0.33**	0.23*	ns	-.47**	-0.10*	-0.22*						
Chicoric acid	0.17	-0.17	ns	-0.29**	-0.03	0.17	0.37**	-0.04	0.51**	-0.46**					
Chlorogenic acid	0.01	-0.10	ns	-0.24*	-0.35**	ns	ns	0.09	0.23*	-0.70**	0.42**				
Rosmarinic acid	0.12	-0.01	ns	-0.38**	-0.38**	ns	ns	-0.13	Ns	-0.58**	0.50**	0.87**			
Quercetin3 *o*-Glucuronide	0.01	-0.02	ns	-0.16	-0.33**	ns	ns	-0.06	Ns	-0.44**	0.40**	0.66**	0.64**		
Kaempferol	ns	ns	ns	Ns	ns	-0.26*	ns	ns	0.21*	ns	ns	0.34**	0.37**	ns	
Quercetin	ns	ns	ns	Ns	ns	ns	ns	ns	0.45**	ns	0.48**	0.44**	0.50**	ns	0.68**

*Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed); ns- not significant

5.5 Relative Growth Rate

Relative growth rate (RGR) of *Centella asiatica* varied significantly within the habitats ($p < 0.001$). Higher RGR was measured in plantlets of shady grassland (0.75 g $g^{-1}mon^{-1}$) and least (0.48 g $g^{-1}mon^{-1}$) in open agricultural land with mean value was 0.64 g $g^{-1}mon^{-1}$ (Fig 5.7).

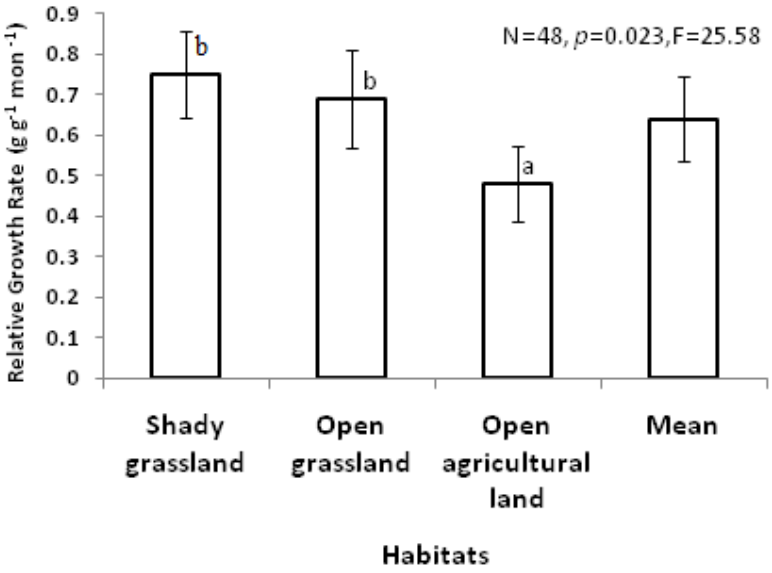


Fig 5.7 Relative growth rate of *Centella asiatica* in different habitats. Values with same letter are not differed significantly. The vertical bars represent the standard errors.

5.6 Phenological Pattern

Phenological events started with emergence of aerial parts of plant from apical bud of perennating creeping stem in growing season (Late Feb. to early March)) when temperature began to rise. At moist open land (i. e. in open agricultural land) plant appeared during the last week of February while in shady places it started to appear only in early summer i.e from last week of March (Fig 5.8). The plant first completed vegetative growth and then started to flower from mid April. At open and sunny sites, the plants began to flower earlier (Fig 5.9). Flowering in open agricultural land started from last week of March, while it was delayed in shady grassland (Fig 5.9). In shady grassland flowering began one month latter than in open agricultural land. April-August was phenologically the most active period when there was flowering, fruiting

and seed germination. During that phenologically active period, 87% of individuals in April and 100% in May were in reproductive stage (flowering and fruiting) in open agricultural land but in shady grassland even during that phenologically active period only 70% individuals were in reproductive stage.

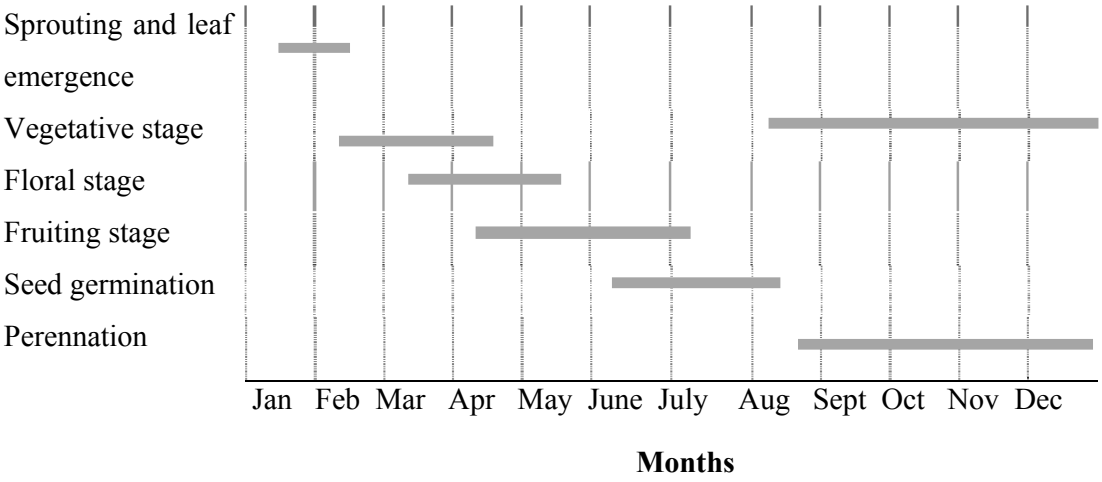


Fig 5.8 Phenological events of *Centella asiatica.*

There was single flowering peak during April-May depending on habitats. Flowering was not synchronized and it continued until October at some habitats. In open agricultural land there was flowering peak in April while it was during June in grassland. Fruiting occurred from May to September, but there was single fruiting peak during May to July depending on habitats. Fruiting frequency varied depending on the habitats. It was higher in open agricultural land, where out of 80% flowering individuals in March, 76% borne fruits in April. But in shady grassland out of 42% flowering individuals in June, 40% of which bore fruits in July (Fig 5.9). Seed maturation nearly took one month. Almost all flowering and fruiting individuals of open agricultural land developed seed while that from shady habitat could not develop seeds.

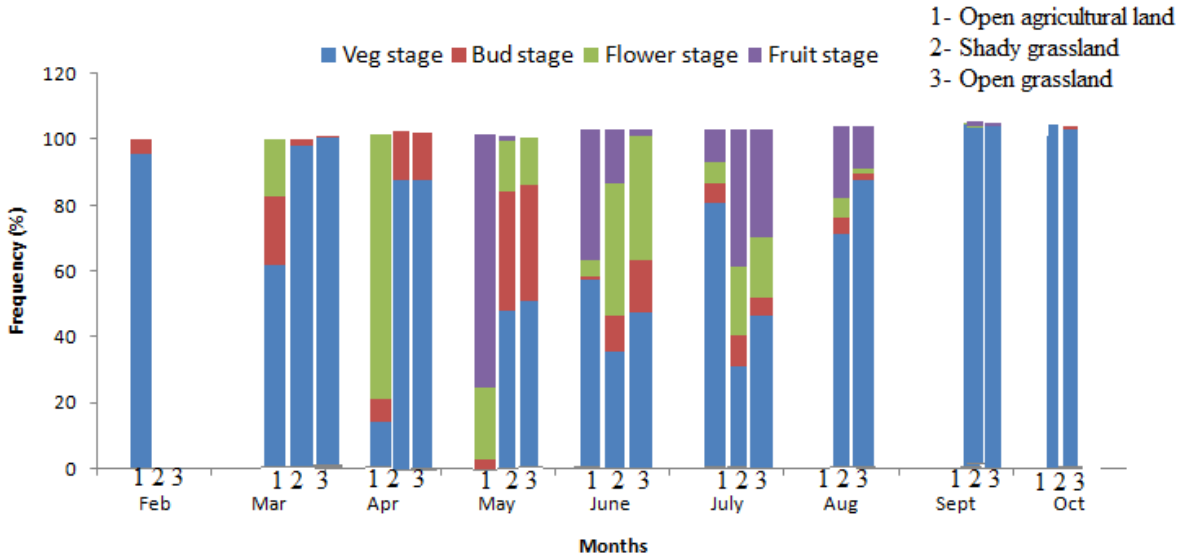


Fig 5.9 Frequency of *Centella asiatica* in various phenophases at three different habitats.

5.7 Germination Ecology

5.7.1 Seed Attributes

Centella asiatica had 9 to 21 seeds per ramet (average 14 .11) (Table 5.4). The seeds of *Centella asiatica* were light brown to dark brown in colour and reniform in shape. The average length and breadth of seeds were 2.49 mm and 1.51 mm, respectively. Average mass of 100 air dried seeds were 0.134 g (Table 5. 4).

5.7.2 Seed Viability

TTC test indicated 82% viability in freshly harvested seeds of *Centella asiatica*. Seeds viability deteriorated as duration of storage increased, and they became non-viable after storage for thirty months (Fig 5.10). Freshly harvested seeds of *C. asiatica* did not germinate even after GA_3 treatment and incubation under different light qualities, whereas 2-3 months old seeds exhibited germination (82%) without pre-treatment at warm environment (25-30°C).

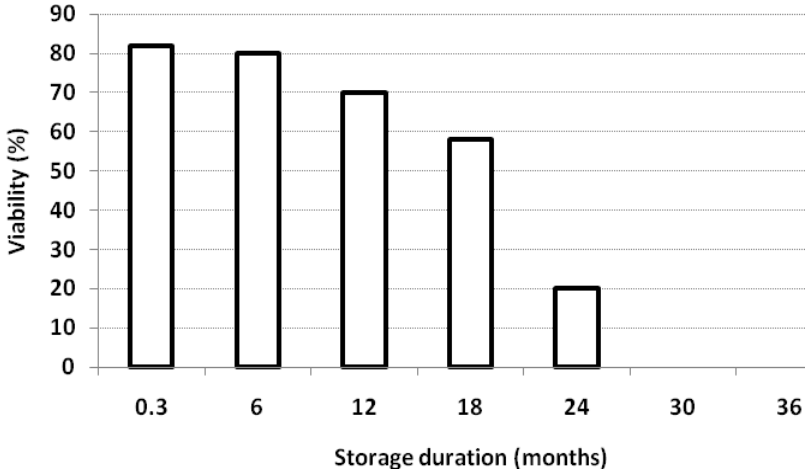


Fig 5.10 Change in seed viability of seeds of *Centella asiatica* during storage.

5.7.3 Effect of Pre-treatments

The effect of ten different pre-treatments on germination of seeds of *Centella asiatica* are shown in Fig 5.11. The germination were 38-70% and differed significantly among the treatments. Only 60ºC hot water shows significant difference in comparision to all other treatments including control Among the different treatments of GA_3, soaking seeds in 10 ppm resulted in highest germination (Fig 5.11) and was followed by 20 ppm. Increasing the concentration did not have any significant effect on germination (Fig 5.11). There seems to be no significant difference between control and pre-treatments except the 60ºC hot water treatment. However GA_3 treatment induced early germination by nearly a week than other treatments where seed germination began from second week after incubation (Table 5.5). Seeds treated with 10% HNO_3 and warm water treatments (40ºC and 60ºC) started germination from third week of incubation, while it started from fourth week in control (distilled water).

5.7.4 Effect of Light

Significant differences (p <0.001) in germination of seeds were observed due to the different wave lengths of white light (blue, red and far red light) (Fig 5.12). It was significantly higher in red light. The seeds germination was inhibited in dark conditions.

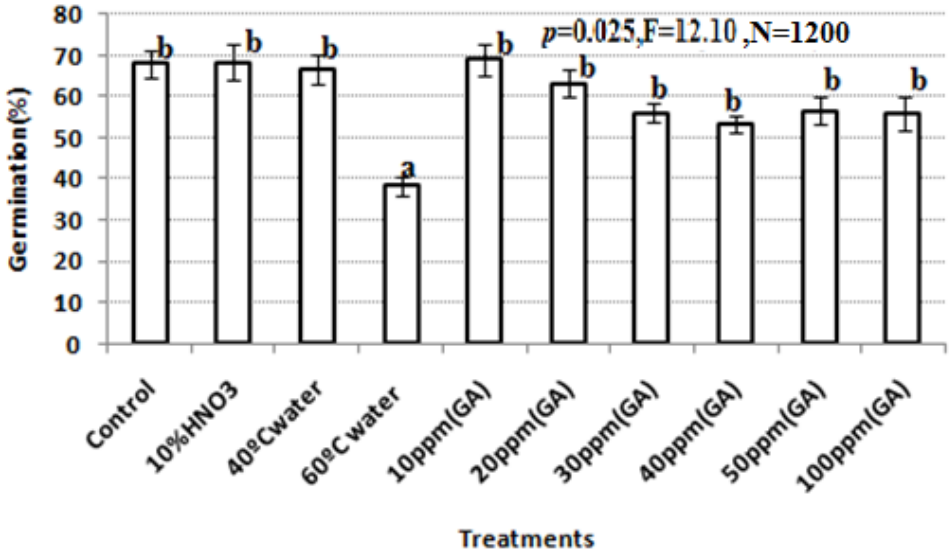


Fig 5.11 Effects of different pre-treatments on seed germination of *C. asiatica*.Values for different pretreatments having the same letter are not significantly different ($P > 0.05$) from the control. The vertical bars represent the standard errors. (Observations taken after 6th week).

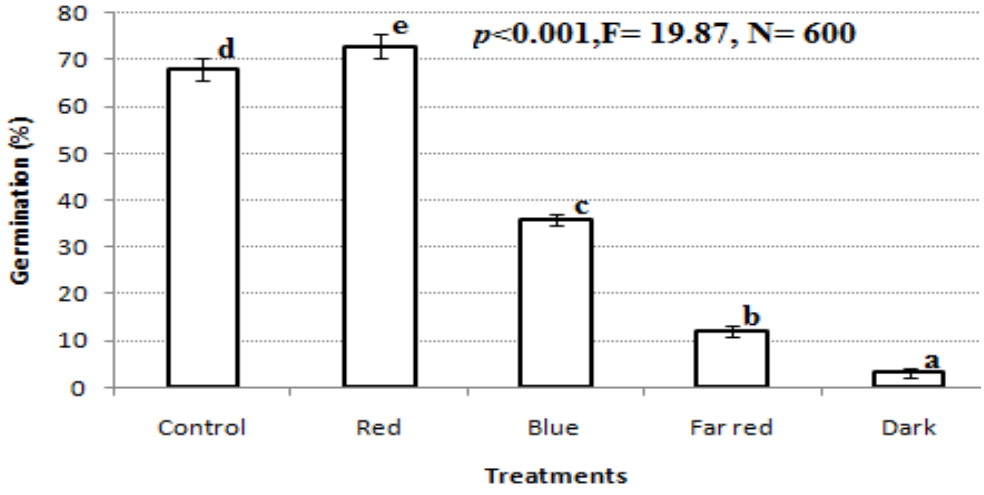


Fig 5.12 Seed germination of *Centella asiatica* in different light (observation at 6th week). Bars with same alphabet above were not significantly different at $p<0.05$. The vertical bars represent the standard errors.

Table 5.4 Seed output and seed characters of *Centella asiatica* at different locations.

Location (District)	Seed mass (g/100)	Seed length (mm)	Seed width (mm)	Seed output/ramet*
Jhapa	$0.13^{cd} \pm 0.01$	$3.41^{i} \pm 0.21$	$1.95^{h} \pm 0.10$	15 ± 4
Sunsari	$0.156^{fg} \pm 0.05$	$2.65^{defg} \pm 0.20$	$1.63^{ef} \pm 0.21$	15 ± 3
Dhankuta	$0.137^{d} \pm 0.02$	$2.73^{fgh} \pm 0.05$	$1.71^{efg} \pm 0.20$	12 ± 3
Hetauda	$0.117^{bc} \pm 0.008$	$2.77^{gh} \pm 0.10$	$1.88^{hi} \pm 0.40$	12 ± 3
Chitwan	$0.20^{h} \pm 0.01$	$3.50^{i} \pm 0.21$	$1.93^{gh} \pm 0.40$	21 ± 3
Lalitpur	$0.100^{a} \pm 0.01$	$2.07^{ab} \pm 0.12$	$1.15^{abc} \pm 0.40$	9 ± 2
Kirtipur	$0.152^{cd} \pm 0.05$	$2.83^{gh} \pm 0.10$	$1.84^{hi} \pm 0.20$	15 ± 3
Matatirtha	$0.109^{a} \pm 0.02$	$2.11^{ab} \pm 0.01$	$1.36^{cd} \pm 0.10$	18 ± 3
Gorkha	$0.096^{a} \pm 0.05$	$1.93^{a} \pm 0.10$	$1.0^{a} \pm 0.10$	12 ± 3
Dhampus	$0.106^{ab} \pm 0.04$	$2.03^{a} \pm 0.10$	$1.05^{ab} \pm 0.10$	9 ± 3
Pokhara	$0.102^{a} \pm 0.05$	$2.06^{ab} \pm 0.30$	$1.14^{abc} \pm 0.10$	18 ± 3
Dang	$0.165^{g} \pm 0.03$	$3.04^{h} \pm 0.10$	$1.93^{gh} \pm 0.10$	15 ± 3
Surkhet	$0.14^{de} \pm 0.01$	$2.63^{defg} \pm 0.30$	$1.79^{ghi} \pm 0.08$	9 ± 3
Banke	$0.138^{d} \pm 0.01$	$2.37^{bcde} \pm 0.12$	$1.35^{cd} \pm 0.10$	18 ± 3
Bardiya	$0.135^{d} \pm 0.03$	$2.42^{cde} \pm 0.40$	$1.72^{efgh} \pm 0.10$	15 ± 2
Kailali	$0.156^{fg} \pm 0.02$	$2.25^{abcd} \pm 0.12$	$1.71^{efg} \pm 0.21$	15 ± 6
Kanchanpur	$0.145^{def} \pm 0.01$	$2.5^{defg} \pm 0.21$	$1.33^{cd} \pm 0.10$	12 ± 2
Mean	0.134 ± 0.02	2.49 ± 0.16	1.51 ± 0.15	14.11 ± 3.12

[a] Samples size (n) for seed mass, length and width ,N=100,* N=30 per population**.** Values with same alphabet were not significantly different at $p<0.05$.

5.7.5 Effect of Salt Stress on Germination

Salinity significantly affected the germination of *Centella asiatica* (p <0.001) (Fig 5.13). At salinities upto 3500 ppm NaCl, germination rate was in range 33-35%. At 4500 ppm germination rate was 31% and at 5500 ppm only 8%. No seed germinated at 6500 ppm salinity. When nongerminated seeds were removed from 6500 ppm and placed in distilled water, there was no germination. This indicates that seeds were

adversely affected by exposure to high salinity and were not able to germinate later on even in favourable conditions.

Table 5.5 Effect of different pre-treatments on germination of seeds of *Centella asiatica* (N=1200).

Treatments	Seed Germination (%)					
	1st week	2nd week	3rd week	4th week	5th week	6th week
Control	0	0	0	4	48	68.00
10% HNO_3	0	0	20	65	65	68.33
40ºC water	0	0	8	40	56	66.66
60ºC water	0	0	8	18	30	38.33
10 ppm GA_3	0	20	65	65	65	70.00
20 ppm GA_3	0	5	35	35	45	63.33
30 ppm GA_3	0	5	35	35	45	56.00
40 ppm GA_3	0	20	40	50	52	53.33
50 ppm GA_3	0	15	50	50	56	56.66
100 ppm GA_3	0	10	20	40	40	56.00

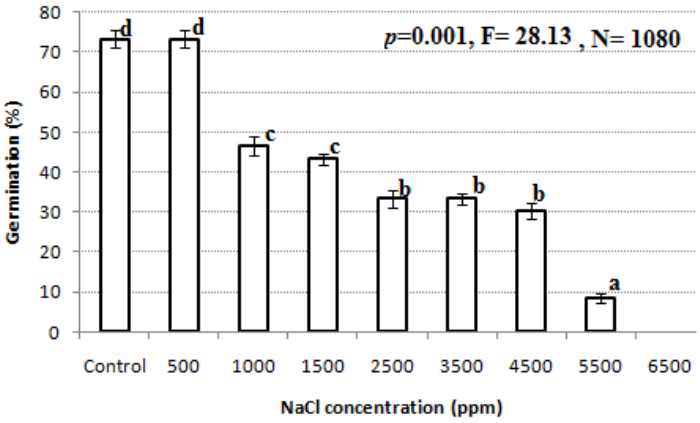


Fig 5.13 Seed germination of *Centella asiatica* in different concentration of NaCl. Bars with same alphabet above were not different significantly at $p = 0.05$. The vertical bars represent the standard errors.

5.7.6 Effect of Leaf Leachates of Alien Plants

The germination of seeds of *Centella asiatica* on leaf leachates of different alien plants are shown in Table 5.6. Highest percentage of germination of seeds was observed at control (78%). Leaf leachates of studied alien plants had inhibitory effects on seed germination of *C. asiatica*. Out of the four plants studied, 10% leaf leachate of *Parthenium hysterophorus* showed significantly higher (p <0.001) inhibitory effects on germination which was followed by 10% leaf leachate of *Ageratum conyzoides* (Table 5.6).

Table 5.6 Effect of leaf leachates of some alien plants on seed germination of *Centella asiatica* (N=1440).

Treatments		Germination (%) ± S.D.					
Plant species	Leachate concentration (%)	1st week	2nd week	3rd weed	4th week	5th week	6th week
Control	-	0	0	5 ± 1	61 ± 1	78 ± 1	78 ± 1
Chromolaena odorata	1	0	0	1 ± 1	35 ± 2	75 ± 2	75 ± 2
	5	0	0	0	20 ± 1	50 ± 1	50** ± 1
	10	0	0	0	26 ± 1	56 ± 1	56** ± 2
Parthenium hysterophorus	1	0	0	0	30 ± 2	63 ± 2	63* ± 2
	5	0	0	0	18 ± 1	51 ± 1	51** ± 1
	10	0	0	0	6 ± 1	20 ± 1	20**±1.0
Ageratum conyzoides	1	0	0	0	3 ± 1	67 ± 2	67* ± 2
	5	0	0	0	1 ± 0	56 ± 1	56** ± 1
	10	0	0	0	0	40 ± 2	40** ± 2
Xanthium strumarium	1	0	0	0	16 ± 1	58 ± 2	65*±2.0
	5	0	0	0	1 ± 0	65 ± 1	65* ± 1
	10	0	0	0	1 ± 0	53 ± 3	53** ± 3

* & ** indicate the significance difference of the value with control at p = 0.05 and 0.001, respectively.

5.8 Effect of Different Environmental Factors

5.8.1 Effect of Moisture Levels

5.8.1.1 Morphological traits

Number of leaves per ramet ranged from 5.62 to 11.11 . Number of leaves per ramet in all treatments differed significantly (p <0.001) (Table 5.7). A significant effect of different moisture levels on leaf morphological characters like petiole length, specific leaf area (SLA) was noted during the experimental period. The longest petiole length (5.67 cm) was observed at 100% FWC and shortest (2.05 cm) at 30%. Similarly highest value of SLA (409 cm^2/g) was obtained at 70% FWC and lowest at 30% FWC. Root length of the plant varied significantly with treatment (p = 0.003) (Table 5.8). Longest root length (10.23 cm) was observed with the plant at 30% FWC. There was significant difference (p <0.001) in number of primary branches, number and length of stolon among the treatments. A significant effect of different levels of water stress on number of flowers per plant was noted (Table 5.8). The highest number of flowers (17.66 flowers/ramet) was obtained at 70% and least number of flowers was at 30% field water capacity.

5.8.1.2 **Leaf nitrogen and chlorophyll content:** Leaf N content ranged from 1.40 to 2.54 % . All treatments exhibited significant difference (p <0.001) in leaf N content. Total chlorophyll content ranged from 9.76 to 25.75 mg/g. There was significant difference in content of total chlorophyll among treatments (p <0.001) (Table 5.7).

5.8.1.3 Dry mass yield

A significant effect of different moisute levels on yield of *Centella asiatica* was also found during the study (Table 5.8). The yield of plant in different moisture levels revealed that the highest yield (1.04 g/ramet) was obtained at 100 %, then at 70% (0.88 g/ramet) and lowest (0.13 g/ramet) at 30% field water capacity.

5.8.2 Effect of Soil Composition

5.8.2.1 Growth and morphological characters

All the measured traits of leaves varied significantly with soil type. There was no significant difference in leaf number among the treatments (Table 5.9). Diameter of

rosette and root length of plants varied significantly (p <0.001) among treatments with longest (18.9 cm) root found in pure sand (Table 5.10).

5.8.2.2 Biomass production

Dry mass of individual plant differed significantly among the soil composition type (p =0.035). It was higher (2.44 g/pl) in sandy loam (S_4) type of soil and least in sand grown plants (Table 5.10).

5.8.3 Effect of Shading

5.8.3.1 Growth and morphological characters

All the measured traits of leaves varied significantly with light intensity (Table 5.11). There was significant difference in leaf number among the treatments with the highest number of leaves in plants grown in 30% shade (Table 5.11). Petiole length ranged from 2.50 cm at full sunlight to 4.63 cm at 70% shading. All treatments differed significantly (p < 0.001) in petiole length. Regarding the stolon length, it was significantly longer (p < 0.001) in plants grown under 70% shading. Specific leaf area (SLA) ranged from 196 cm^2/g at full sunlight to 1536 cm^2/g at 70% shading . The difference in SLA among the treatments was significant (p = 0.002) (Table 5.11). Regarding leaf chlorophyll concentration, an increment in these photoreceptors was observed with the increase of shading, reaching the highest values in plants grown under 70% shading. There was significant difference (p = 0.001) in leaf N concentration ranging from 1.73% at full light to 2.66% at 70% shading .Number of primary branches per ramet also differed significantly (p <0.001) among treatments, a higher number being in treatment with 30% shading (Table 5.12). Longest root length (13.7 cm) was obtained in plants grown in full sunlight.The number of flowers per ramet ranged from 17.90 in plants grown in full sunlight to 0.90 in plants grown in 70% shading condition.

5.8.3.2 Biomass production

There was significant effect of light intensity on biomass production of *Centella asiatica*. It was significantly higher in partial shade (30% and 50 % shade) than in other treatment (Table 5.12, p <0.001).

Table 5.7 Leaf characters (mean ± SD) of *Centella asiatica* in different moisture level measured as field water capacity (FWC) of soil. For each parameter significant difference between mean among different sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[A]	T_1 (125 % FWC)	T_2 (100% FWC)	T_3 (70% FWC)	T_4 (30% FWC)	F value	P value
Number of leaves/ramet*	$6.92^{a} \pm 3.42$	$11.11^{b} \pm 5.52$	$6.66^{a} \pm 3.85$	$5.62^{a} \pm 0.87$	10.27	<0.001
Petiole length (cm)**	$4.12^{b} \pm 0.78$	$5.67^{b} \pm 0.8$	$3.21^{a} \pm 0.70$	$3.05^{a} \pm 0.60$	31.40	0.001
Dry mass of individual leaf (g)**	$0.023^{b} \pm 0.001$	$0.021^{b} \pm 0.002$	$0.027^{c} \pm 0.001$	$0.012^{a} \pm 0.003$	21.38	0.000
SLA cm^2/gm**	$218.0^{a} \pm 34.0$	$262.0^{ab} \pm 113.5$	$409.0^{b} \pm 43.2$	$137.0^{a} \pm 22.0$	4.21	0.008
Leaf N (%)***	$1.68^{a} \pm 0.07$	$2.25^{b} \pm 0.42$	$2.54^{b} \pm 0.028$	$1.40^{a} \pm 0.29$	21.19	0.000
Total chlorophyll(mg/g)****	$10.15^{a} \pm 6.09$	$25.75^{b} \pm 0.18$	$25.43^{b} \pm 0.089$	$9.76^{a} \pm 0.010$	9740.612	0.000

[A] Sample size (N) of each treatment *N = 30 ** N = 90 *** N = 20, ****N=5 .

Table 5.8 Growth traits and yield (mean ± SD) of *Centella asiatica* in different moisture level measured as field water capacity (FWC) of soil. For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA). (Sample size for each treatment, N=30).

Attributes	T_1 (125% FWC)	T_2 (100% FWC)	T_3 (70% FWC)	T_4 (30% FWC)	F value	P value
Number of primary branches / ramet	$5.0^{c} \pm 2.25$	$5.55^{c} \pm 1.59$	$2.87^{b} \pm 1.39$	$1.33^{a} \pm 0.48$	37.92	<0.001
Rosette diam (cm)	$10^{b} \pm 1.9$	$8.8^{b} \pm 3.27$	$5.21^{a} \pm 1.59$	$3.11^{a} \pm 0.99$	27.72	0.001
Number of stolons	$2.62^{b} \pm 1.01$	$4.44^{c} \pm 1.28$	$3.03^{b} \pm 1.1$	$1.66^{c} \pm 1.27$	24.13	0.001
Stolon length (cm)	$5.08^{b} \pm 0.52$	$5.21^{b} \pm 0.46$	$3.84^{a} \pm 0.73$	$3.8^{a} \pm 0.52$	46.91	0.001
Root length (cm)	$8.20^{b} \pm 0.67$	$8.82^{b} \pm 1.1$	$7.10^{a} \pm 0.87$	$10.23^{c} \pm 1.2$	4.91	0.003
Number of flowers per node	$5.11^{a} \pm 3.3$	$6.37^{b} \pm 10.13$	$17.66^{c} \pm 10.13$	0.0	58.5	0.001
Dry mass/plant (g)	$0.21^{b} \pm 0.12$	$1.04^{c} \pm 0.81$	$0.88^{c} \pm 0.11$	$0.13^{a} \pm 0.09$	71.21	<0.001
Moisture content of plant(%)	$88.15^{d} \pm 3.18$	$77.52^{c} \pm 1.56$	$68.12^{b} \pm 3.35$	$46.74^{a} \pm 0.65$	15.54	0.000

Table 5.9 Mean (±SD) leaf attributes of *Centella asiatica* in different soil textural type. For each parameter significant difference between mean among different treatments are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[A]	100% clay (S_1)	20% sand + 80% clay (S_2)	40% sand + 60% clay (S_3)	60% sand + 40 % clay (S_4)	80% sand + 20% clay (S_5)	100% sand (S_6)	F value	P value[b]
No. of leaves*	$3.66^{a} \pm 0.5$	$4.31^{a} \pm 0.28$	$3.25^{a} \pm 0.55$	$5.29^{a} \pm 0.8$	$4.65^{a} \pm 0.52$	$3.65^{a} \pm 0.24$	1.499	0.197
Petiole length (cm)**	$11.51^{bc} \pm 4.77$	$12.54^{c} \pm 6.83$	$12.69^{c} \pm 4.95$	$12.38^{c} \pm 3.57$	$9.16^{ab} \pm 2.88$	$8.22^{a} \pm 2.52$	3.604	**0.005**
Dry mass of leaf (g)**	$0.08^{a} \pm 0.03$	$0.08^{a} \pm 0.04$	$0.07^{a} \pm 0.045$	$0.05^{a} \pm 0.02$	$0.11^{b} \pm 0.24$	$0.09^{a} \pm 0.09$	2.52	**0.034**
SLA (cm^2/g)**	$128.0^{a} \pm 64$	$235.0^{c} \pm 193.0$	$284.0^{ab} \pm 71.0$	$292.0^{c} \pm 44.0$	$194.0^{b} \pm 35.0$	$152.0^{a} \pm 57.0$	3.68	**0.004**
Leaf N (%) ***	$1.69^{a} \pm 0.31$	$2.11^{b} \pm 0.18$	$2.1^{b} \pm 0.107$	$2.16^{b} \pm 0.0195$	$1.65^{a} \pm 0.12$	$1.26^{a} \pm 0.18$	67.55	**0.000**
Total chlorophyll (mg/g)****	$17.14^{b} \pm 0.07$	$25.48^{c} \pm 0.17$	$23.75^{c} \pm 0.18$	$20.43^{c} \pm 0.08$	$19.35^{b} \pm 0.20$	$6.23^{a} \pm 0.00$	9740	**<0.001**

[A] Sample size (N) for each treatment: *N = 30 ** N = 90 *** N = 20, **** N=5; [b]Bold number indicates significant difference among the mean.

Table 5.10 Growth traits and yield of *Centella asiatica* in different soil textural group. For each parameter significant difference between mean among the treatments are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes*	100 % clay (S_1)	20 % sand + 80% clay (S_2)	40% sand + 60% clay (S_3)	60% sand + 40 % clay (S_4)	80% sand + 20% clay (S_5)	100 % sand (S_6)	F value	P value
Number of primary Branch*	$2.73^{b} \pm 1.33$	$3^{b} \pm 0.96$	$2.87^{b} \pm 1.25$	$2.76^{b} \pm 1$	$2.7^{b} \pm 0.92$	$1.85^{a} \pm 0.7$	2.86	0.018
Rosette diam. (cm)*	$12.4^{d} \pm 2.13$	$15.28^{c} \pm 3.33$	$18.18^{d} \pm 3.15$	$19.52^{c} \pm 2.29$	$13.25^{b} \pm 1.86$	$10.5^{a} \pm 3.03$	17.19	< 0.001
Number of stolons*	$4.66^{a} \pm 3.24$	$3.43^{a} \pm 1.26$	$4.93^{a} \pm 3.43$	$4.94^{a} \pm 2.16$	$5.05^{a} \pm 2.01$	$4.7^{a} \pm 1.62$	1.052	0.392
Stolon length (cm) *	$11.81^{a} \pm 1.69$	$22.14^{a} \pm 45.04$	$12.28^{a} \pm 1.93$	$11.77^{a} \pm 1.44$	$10.79^{a} \pm 1.89$	$9.32^{a} \pm 1.25$	1.12	0.357
Root length (cm)*	$7.64^{a} \pm 1.60$	$9.97^{ab} \pm 2.10$	$10.93^{ab} \pm 2.8$	$10.97^{ab} \pm 3.80$	$12.1^{b} \pm 7.20$	$18.9^{c} \pm 5.10$	10.23	< 0.001
Number of flowers/node*	$9.1^{a} \pm 2.20$	$12.3^{ab} \pm 3.90$	$18.4^{b} \pm 4.17$	$21^{b} \pm 4.21$	$12.7^{ab} \pm 3.30$	$15.2^{ab} \pm 3.90$	1.75	0.131
Dry mass of individual plant (g)*	$1.79^{a} \pm 0.62$	$2.07^{ab} \pm 1.40$	$2.24^{b} \pm 1.10$	$2.44^{b} \pm 1.04$	$2.11^{ab} \pm 1.04$	$1.38^{a} \pm 0.68$	2.51	0.035
Moisture content of plant*	$73.45^{a} \pm 1.20$	$72.61^{b} \pm 3.25$	$74.42^{a} \pm 1.12$	$71.55^{ab} \pm 2.21$	$64.7^{b} \pm 1.23$	$43.75^{b} \pm 1.2$	12.36	< 0.001

* Sample size (N) for each treatment: * N=40

Table 5.11 Leaf characters (mean ± SD) of *Centella asiatica* in different light conditions. For each parameter the significant difference between mean among the treatments are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[A]	Full light (0% shade)	30% Shade	50 % Shade	70 % Shade	F value	P value
No. of leaves*	$20.3^{ab} \pm 9.6$	$22.7^{b} \pm 3.8$	$16.6^{a} \pm 8.4$	$12.6^{a} \pm 4.6$	4.456	0.006
Petiole length (cm)**	$2.50^{a} \pm 0.6$	$2.60^{b} \pm 0.5$	$4.60^{c} \pm 0.9$	$4.63^{c} \pm 0.8$	54.17	0.000
SLA (cm^2/g)**	$196.0^{a} \pm 30.0$	$667.0^{b} \pm 192$	$873.0^{b} \pm 154$	$1536^{c} \pm 146$	5.429	0.002
Leaf N (%) ***	$1.73^{a} \pm 0.23$	$2.38^{b} \pm 0.5$	$2.41^{bc} \pm 0.28$	$2.66^{c} \pm 0.22$	35.31	0.000
Total chlorophyll (mg/g)****	$11.28^{a} \pm 0.0$	$17.14^{b} \pm 0.00$	$17.44^{b} \pm 0.00$	$19.13^{b} \pm 0.00$	25.15	<0.001

[A] Sample size (N) for each treatment: *N = 30, **N = 90,*** N = 20, **** N= 5

5.8.4 Effect of Different Integrated Manuring

5.8.4.1 Morphological traits

Different combinations of manuring significantly affected the leaf characteristics of *Centella asiatica*. The highest number of leaves per ramet was observed in treatment T_3 (50/50; Urea /FYM) followed by T_2 (75/25; Urea /FYM) and T_4 (25/75; Urea/FYM). Longest petiole and relatively higher SLA were observed in treatment T_3 (50/50; Urea /FYM) followed by T_2 (75/25; Urea /FYM) and T_4 (25/75; Urea /FYM). The highest number of flowers per ramet (16.82) was also obtained in T_3 (50/50; Urea /FYM) (Table 5.14). There was significant difference (p <0.001) in number of primary branches, stolon length and number of stolons among the treatments. There was no significant difference in petiole length, stolon length, SLA and number of primary branches among the integrated manuring, but it was significantly higher than the individual application and control (Tables 5.13 & 5.14).

Table 5.12 Growth traits and yield (mean ± SD) of *Centella asiatica* in different light conditions. For each parameter significant difference between mean among the treatments are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA). Sample size for each treatment (N) =40.

Attributes	Full light (0% shade)	30 % Shade	50 % Shade	70% Shade	F value	P value
Number of prim. branches	$3.80^{b} \pm 2.4$	$5.60^{c} \pm 1.5$	$3.60^{b} \pm 2.1$	$2.0^{a} \pm 0.7$	13.218	<0.001
Ramet diam (cm)	$11.50^{b} \pm 2.9$	$8.50^{a} \pm 2.5$	$8.80^{a} \pm 3.6$	$6.10^{a} \pm 3.3$	10.52	<0.001
Number of stolons	$2.80^{b} \pm 1.1$	$7.60^{c} \pm 0.8$	$3.20^{b} \pm 0.9$	$2.0^{a} \pm 0.7$	160.182	<0.001
Length of stolon (cm)	$2.87^{a} \pm 0.44$	$4.92^{b} \pm 0.69$	$4.93^{b} \pm 0.68$	$5.10^{b} \pm 0.84$	182.726	<0.001
Number of flowers per node	$17.90^{c} \pm 11.5$	$10.70^{b} \pm 11.3$	$9.60^{b} \pm 9.8$	$0.90^{a} \pm 2.2$	10.220	<0.001
Dry mass of individual plant (g)	$0.90^{b} \pm 0.24$	$1.15^{b} \pm 0.25$	$1.05^{b} \pm 0.25$	$0.76^{a} \pm 0.09$	79.39	<0.001
Root length (cm)	$13.70^{b} \pm 2.1$	$12.81^{b} \pm 1.4$	$8.20^{a} \pm 1.0$	$6.80^{a} \pm 2.3$	16.25	<0.001
Root mass (g)	$0.15^{b} \pm 0.02$	$0.12^{b} \pm 0.04$	$0.08^{a} \pm 0.01$	$0.07^{a} \pm 0.02$	19.69	<0.001

5.8.4.2 Leaf nitrogen (N) and chlorophyll content

Leaf N content ranged from 1.22 to 2.2 %. All treatments differed (p <0.001) significantly in leaf N content. Total chlorophyll content ranged from 4.6 to 12.6 mg/g . There was significant difference in total chlorophyll content among treatments (p <0.001) (Table 5.13).

5.8.4.3 Biomass production

A significant effect of various combinations of manuring on biomass production of *C. asiatica* was found during the study (Table 5.14). Dry herb yield of the plant was significantly higher in all manuring combination than in control. The dry mass of the individual plant was around 7 times higher in integrated manuring system (T_3 treatment; 50/50; Urea/FYM) than in control. It was 1.5 and 5 times higher than in application of 100% urea and 100 % FYM, respectively.

5.9 Nutrient Content

Leaf N content ranged from 1.52 to 1.95% (average 1.76%) (table 5.15). Leaf N content differed significantly (p <0.001) along *C. asiatica* habitats.

Soil pH ranged from 5.57(open agrasland land) to 5.83 (partially shaded grassland). There was significant difference (p <0.001) in soil pH among the habitats. Soil N content ranged from 0.16 to 0.20 % (average 0.182 %) and there was significant difference (p <0.001) among three habitats (Table 5.15). Soil organic carbon (OC) ranged from 2.55 % at open agricultural land to 4.26 % at shady land with average 3.16 % for all sites. Mean soil organic matter (OM) was 5.64 %. There was significant difference in soil OM and OC among the sites (Table 5.15). C/N ratio ranged from 15.93 at open agricultural land to 21.3 at shady grassland. There was no significant difference in C/N ratio among habitats**.**

Nirogen content of leaves and soil did not vary consistently with altitude (Figs 5.14 & 5.15). There was significant variation (p =0.001) of leaf nitrogen and soil nutrient contents in different ecological region (Fig 5.16). Higher amount of nitrogen contents were observed in samples of eastern Nepal than in others.

Table 5.13 Leaf attributes (mean ±SD) of *Centella asiatica* in different combination of urea and farm yard manure. For each parameter significant difference between mean among different treatment are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA). (Treatments; T_1: 100% Urea; T_2:75% Urea, 25% FYM; T_3: 50% Urea, 50% FYM; T_4: 25% Urea, 75% FYM; T_5: 100% FYM; T_6: control (no manure).

Attributes[A]	T_1	T_2	T_3	T_4	T_5	T_6	F value	P value
No. of leaves per ramet*	$5.8^{a} \pm 1.6$	$6.8^{ab} \pm 1.0$	$7.2^{b} \pm 1.8$	$6.5^{b} \pm 1.5$	$5.4^{a} \pm 1.6$	$4.4^{a} \pm 1.8$	2.38	0.043
Petiole length (cm)**	$5.4^{ab} \pm 0.9$	$6.7^{ab} \pm 0.8$	$8.2^{b} \pm 1.1$	$6.9^{ab} \pm 0.4$	$5.7^{a} \pm 0.4$	$2.2^{a} \pm 0.5$	4.17	0.002
Dry mass of individual leaf (g)**	$0.037^{b} \pm 0.01$	$0.027^{b} \pm 0.00$	$0.023^{a} \pm 0.05$	$0.025^{a} \pm 0.06$	$0.03^{b} \pm 0.01$	$0.038^{b} \pm 0.01$	4.56	0.001
SLA (cm^2/g)**	$204^{a} \pm 25$	$345^{b} \pm 35$	$406^{b} \pm 15$	$331^{ab} \pm 25$	$204^{a} \pm 17$	$197^{a} \pm 36$	1.23	0.036
Leaf N (%) ***	$1.6^{c} \pm 0.07$	$1.58^{c} \pm 0.07$	$2.2^{d} \pm 0.24$	$1.79^{d} \pm 0.24$	$1.22^{a} \pm 0.10$	$1.37^{b} \pm 0.10$	46.63	<0.001
Total Chlorophyll (mg/g) *****	$7.25^{b} \pm 0.88$	$11.41^{d} \pm 0.53$	$12.6^{e} \pm 0.16$	$11.5^{f} \pm 0.20$	$8.33^{c} \pm 0.37$	$4.60^{a} \pm 0.20$	295.4	<0.001

[A]Sample size *N=40 ,**N=90 , *** N=20, ***** N=5.

Table 5.14 Growth traits and yield (mean± SD) of *Centella asiatica* in different combination of urea and farm yard manure. For each parameter, significant difference between mean among different treatments are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P value were obtained by one way analysis of variance (ANOVA). (Treatments; T_1: 100% Urea; T_2:75% Urea, 25% FYM; T_3: 50% Urea, 50% FYM; T_4: 25% Urea, 75% FYM; T_5: 100% FYM; T_6: control (no manure). Sample size for all attributes was 40.

Attributes	T_1	T_2	T_3	T_4	T_5	T_6	F value	P value
No. of primary branch	$9.47^{b} \pm 1.40$	$8.68^{b} \pm 1.02$	$9.33^{c} \pm 5.24$	$8.16^{b} \pm 1.33$	$5.75^{a} \pm 1.11$	$4.35^{a} \pm 0.70$	13.52	<0.001
No of stolon	$4.86^{ab} \pm 1.13$	$6^{c} \pm 1.81$	$6.2^{c} \pm 1.81$	$5.59^{bc} \pm 1.09$	$4.56^{a} \pm 0.76$	$7.46^{d} \pm 0.97$	9.79	<0.001
Diam of ramet (cm)	$8.06^{bc} \pm 1.27$	$9.52^{d} \pm 1.53$	$9.7^{d} \pm 2.01$	$9.42^{d} \pm 1.53$	$7.87^{b} \pm 1.10$	$4.01^{a} \pm 1.5$	19.5	<0.001
Stolon length (cm)	$7.39^{c} \pm 0.18$	$8.39^{d} \pm 0.88$	$8.98^{d} \pm 1.44$	$8.27^{d} \pm 0.57$	$6.71^{b} \pm 0.89$	$5.71^{a} \pm 1.03$	21.01	<0.001
Root length (cm)	$4.14^{a} \pm 0.98$	$5.04^{ab} \pm 0.78$	$5.87^{a} \pm 0.95$	$6.68^{b} \pm 1.03$	$5.73^{a} \pm 1.08$	$7.23^{b} \pm 1.46$	8.73	<0.001
No. of flowers/ramet	$10.38^{a} \pm 1.84$	$16.52^{c} \pm 2.89$	$16.82^{c} \pm 2.82$	$14.33^{cd} \pm 2.04$	$12.37^{ab} \pm 2.11$	$8.82^{bc} \pm 1.89$	5.46	<0.001
Dry mass of individual plant (g)	$5.74^{b} \pm 1.27$	$8.62^{c} \pm 1.74$	$8.68^{c} \pm 1.26$	$7.07^{c} \pm 1.58$	$1.7^{a} \pm 1.44$	$1.23^{a} \pm 0.81$	34.53	<0.001

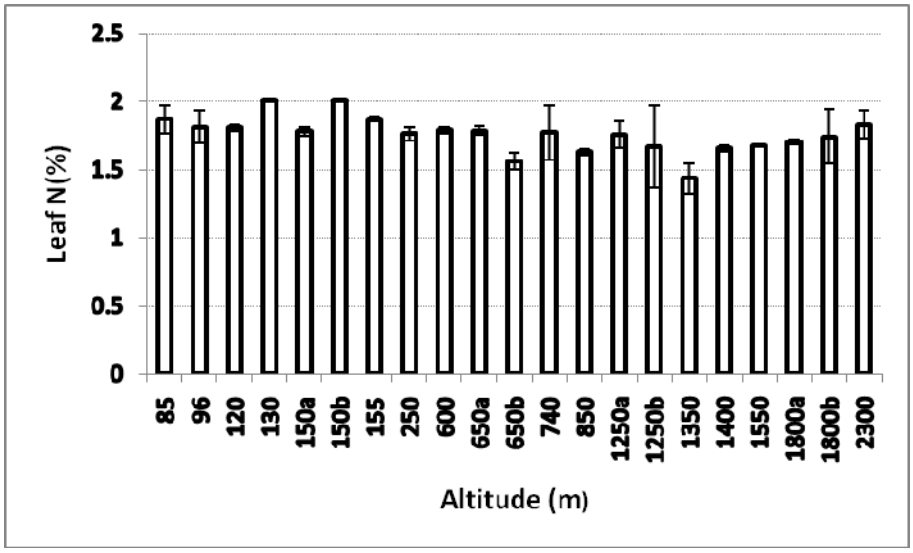


Fig 5.14 Leaf nitrogen (N=540) in leaves of *Centella asiatica* at different altitude. The vertical bars represent the standard errors.

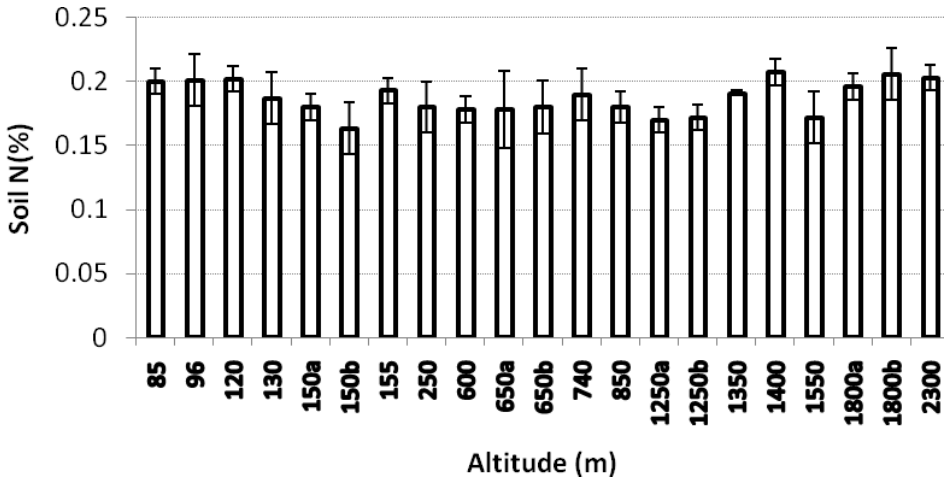


Fig 5.15 Soil Nitrogen (N=540) content at habitat of *Centella asiatica* at different altitude. The vertical bars represent the standard errors.

Table 5.15 Leaf nitrogen (N) content and soil nutrients (nitrogen and organic carbon) in *Centella asiatica* grown in different habitats. For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes	Partial shaded grassland	Open grassland	Open agricultural land	N	Mean	F value	P value
Leaf N (%)	1.95 ^{c} ±0.74	1.65 ^{b} ±0.63	1.52 ^{a} ±0.73	630	1.76±0.72	23.55	< 0.001
Soil pH	5.83 ^{b} ±0.94	5.57 ^{a} ±1.02	5.69 ^{a} ±0.7	540	5.63±0.7	8.62	< 0.001
Soil N (%)	0.20 ^{b} ±0.07	0.18 ^{b} ±0.10	0.16 ^{a} ±0.07	540	0.18±0.08	14.97	< 0.001
Soil OM (%)	7.38 ^{b} ±1.21	5.14 ^{b} ±1.86	4.41 ^{a} ±2.21	540	5.64 ±1.76	19.89	0.032
Soil OC (%)	4.26 ^{b} ±1.21	2.97 ^{b} ±1.26	2.55 ^{a} ±1.3	540	3.16±1.26	20.49	0.041
CN ratio	21.3 ^{a} ±2.65	16.5 ^{a} ±2.34	15.93 ^{a} ±4.32	540	17.91±3.	0.95	0.386

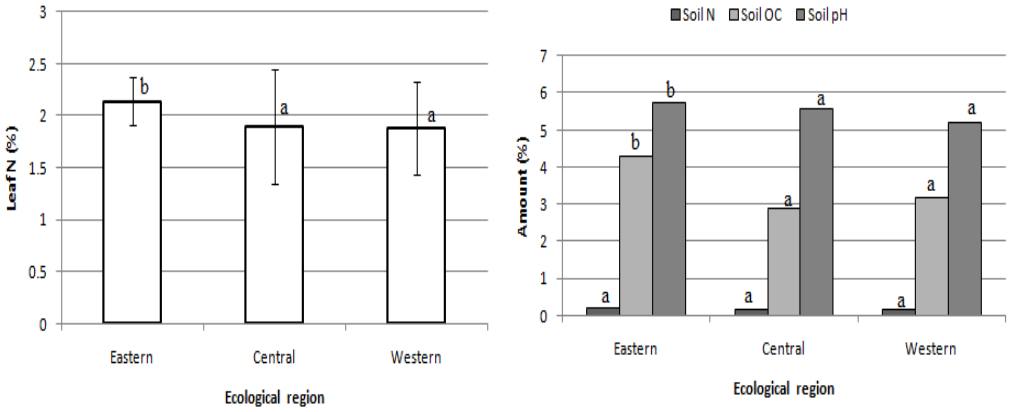


Fig 5.16 Leaf N (N=630) and Soil nutrient (N=540) content of *Centella asiatica* habitats at different ecological regions of Nepal Values with same letter are not differed significantly. The vertical bars represent the standard errors.

5.10 Phytochemical Analysis

5.10.1 Phytochemical constituents of *Centella asiatica* in wild samples

Phytochemical constituents of 21 populations of *Centella asiatica* has been presented in Table 5.16. Highest amount of asiaticoside (8.13% d.w.) was found in samples from Gorkha district and it was followed by samples from Chitwan (4.75% d.w). Asiatic acid could not be detected in most of the collected samples. HPLC analysis revealed important variability in the content of analyzed chemical components among the samples in different habitats (Table 5.17). Phytochemical components varied differently in different habitats depending on the nature of chemical constituents. All chemical components analyzed were higher in open agricultural land. Asiaticoside concentration ranged from 1.41% (d.w.) in shady grassland to 1.91 % (d.w.) in open agricultural land (Table 5.17). There was significant difference in asiaticoside content ($p = 0.003$) among the samples from different habitats. Regarding asiatic acid, chicoric acid, chlorogenic acid and quercetin there was no significant difference in the samples from different habitats.

Considering the mean concentration of the secondary metabolites (bioactive compounds) in the samples collected in the three different regions, the samples from central Nepal contained the higher amount of all secondary metabolites analysed in this study (Table 5.18). The asiaticoside ranged from 0.24 to 8.13 % with a mean value of 1.88% (d.w) in samples collected from central Nepal. In samples from eastern Nepal it ranged from 1.06 to 1.53% with a mean value of 1.20%. Asiaticoside content in samples collected from western Nepal showed a great variability ranging from the non detectable amount (<0.05%) to 2.99% with mean value of 1.05%. Samples from central Nepal presented the higher amount of asiatic acid, chicoric acid, kaempferol and quercetin . The amount of asiatic acid in all the plants collected were very low. Small amount of the phenolic constituents were detected in all the samples with small differences considering geographic origin. Samples from central Nepal contained relatively higher level of phenolics than the others (Table 5.18). Significant differences were observed in chlorogenic acid and quercetin -3-O-glucuronide contents among the samples. In samples from central and western Nepal, chlorogenic acid content was six and three times higher than the eastern, with an average of 0.52 and 0.26%,

respectively. The amount of quercetin -3-O-glucuronide was higher in the samples from central Nepal with mean value of 1.32%. The amount of kaempferol, quercetin and rosmarinic acid was almost similar in all the analyzed samples.

Correlations were observed between altitude of the sampling sites and the amount of asiaticoside and quercetin -3-O-glucuronide. Samples collected from 150 to 600 m asl seemed to have the higher asiaticoside content. Altitude of collection and asiaticoside content was inversely related with higher content of triterpene in samples collected from 150 to 600 masl. Thus, a negative correlation was observed between altitude and asiaticoside content (r= - 0.53, p= 0.015)) while the contrary was for quercetin -3-O-glucuronide content (r = 0.63, p=0.003) (Fig 5.17).

5.10.2 Phytochemical constituents of *Centella asiatica* in transplanted samples

The germplasms used for the phytochemical analyses of wild samples were also used for transplantation experiment in Kathmandu with subsequent phytochemical analyses. Concentration of the selected secondary metabolites has been presented in Table 5.19. The amount of the secondary metabolites was remarkably lower in transplanted samples if compared with wild. For example, the mean concentration of asiaticoside in wild sampled collected from central Nepal was 1.88 % whereas the corresponding value for transplanted ones was 0.68 %, i.e 2.8 times higher in wild samples. Likewise, 9.45 times higher chlorogenic acid in wild samples than in transplanted samples.

The amount of asiatic acid was relatively higher in transplanted samples in comparison to the wild ones (Tables 5.18 & 5.19). Furthermore, significant difference was observed in concentration of quercetin 3-O-glucuronide among the transplanted samples (Table 5.19). The other remaining components such as chicoric acid, quercetin and chlorogenic acid were detected in small amounts in all samples as observed for the wild ones. Kaempferol and rosmarinic acid in transplanted samples were below the level of detection.

Table 5.16. Phytochemical constituents of *Centella asiatica* at different populations (Wild samples). (N= 216). For each chemical constituent significant difference between mean among the populations are indicated by different letters (Duncan homogeneity test, α = 0.05).

Populations Site (District)	Asiatico side	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin -3-O- glucuronide	Kaempferol	Quercetin
Phedim (Ilam)	1.12^{cd} ±0.08	nd	nd	nd	nd	0.35^{b} ±0.01	0.037^{b} ±0.0	0.037^{b}±0.05
Chandragadi) (Jhapa	1.52^{d} ± 0.27	nd	nd	nd	nd	0.185^{b} ±0.01	0.03^{b} ±0.0	0.036^{b}±0.00
Inaruwa (Sunsari)	1.79^{e} ±0.42	nd	0.16^{b} ± 0.22	0.14^{b} ±0.04	0.06^{a} ±0.01	0.124^{b} ±0.04	0.37^{c} ±0.04	0.38^{c} ± 0.00
Hille (Dhankuta)	1.62^{de} ±0.42	0.31^{b} ±0.01	0.05^{a}±0.03	0.05^{a}±0.02	0.07^{a} ± 0.01	0.27^{b} ± 0.05	0.38^{c} ± 0.00	0.39^{c} ±0.006
Hetauda (Makwanpur)	0.76^{bc} ±0.12	nd	0.02^{a} ±0.00	0.03^{a} ± 0.00	0.035^{a} ± 0.00	0.25^{b} ±0.01	0.33^{c} ± 0.01	0.031^{a}± 0.005
Bharatpur (Chitwan)	4.75^{h} ±0.77	nd	0.07^{a} ± 0.02	0.29^{b} ± 0.06	0.18^{b} ±0.05	0.84^{c}± 0.05	0.38^{c} ± 0.00	0.38^{c}± 0.006
Daman(Makwanpur)	0.20^{ab} ±0	0.45^{b}±0.37	0.08^{a} ± 0.04	0.03^{a} ± 0.03	0.035^{a} ± 0.0	0.88^{c} ± 0.12	0.019^{a} ± 0.00	0.031^{a}±0.001
Godavari (Lalitpur)	0.53^{abc} ±0.05	nd	0.05^{a}±0.00	2.09^{d}±0.34	0.22^{c}±0.03	2.2^{d}±0.22	0.37^{c}±0.007	0.38^{c}±0.007
Kirtipur(Kathmandu)	0.42^{ab} ±0.04	nd	0.05^{a}±0.0	0.33^{c} ± 0.02	0.21^{bc}±0.02	2.2^{d}±0.15	0.37^{c}±0.013	0.37^{c} ±0.01
Matatirtha(Kathmandu)	0.52^{abc} ±0.01	nd	0.06^{a}±0.01	0.32^{c}±0.01	0.19^{b}±0.03	1.96^{d}±0.34	0.37^{c}±0.00	0.37^{c} ±0.01
Palungtar (Gorkha)	8.13^{i} ±1.19	nd	0.1^{b}±.01	0.36^{c}±.01	0.34±.01	1.28^{d}±.09	0.29^{c}±0.01	0.38^{c} ±0.005
Basisahar (Lamjung)	0.24^{ab} ±0.04	0.027^{a}±0.02	0.01^{a}±0.001	nd	0.02^{a}±0.00	0.33^{c}±0.03	0.3^{c}±0.027	0.021^{a}±0.002
Dhampus (Kaski)	1.64^{de} ±0.16	0.27^{b}±0.02	0.06^{a}± 0.01	0.23^{b}±0.03	0.17^{b}±0.03	1.85^{d}±.18	0.36^{c}±0.01	0.36^{c} ±0.008
Pokhara (Kaski)	0.54^{abc} ±0.06	0.26^{b}±0.22	0.03^{a}±0.00	0.04^{a}±0.00	0.02^{a}±0.01	0.31^{b}±0.04	0.34^{c}±0.01	0.37^{c} ±0.011
Amnpchaur (Pyuthan)	0.63^{bc} ±0.08	0.6^{b}±0.038	0.04^{a}±0.001	0.70±0.04	0.27^{c}±0.03	0.30^{b}±0.01	0.37^{c}±0.00	0.37^{c} ±0.005
Lamahi (Dang)	1.75^{e} ±.55	nd	0.15^{b}±0.19	0.17^{b}±0.01	0.07^{a}±0.01	0.58^{b}±0.08	0.39^{c}±0.02	0.39^{c} ±0.01
Birendranagar (Surkhet)	nd	nd	0.05^{a}±0.006	0.10^{b}±0.02	0.03^{a}±0.06	0.33^{b}±0.021	nd	nd
Kohalpur (Banke)	3.08^{f} ±0.29	nd	0.06^{a}±0.006	0.29^{b}±0.06	0.06^{a}±0.07	0.73^{c}±0.08	0.38^{c}±0.00	0.38±0.007
Magaragadi (Bardiya)	3.39^{f} ±0.73	nd	0.45^{c}±0.44	0.24^{b}±0.05	0.26^{c}±0.04	0.68^{b}±0.15	0.38^{c}±0.01	0.38^{c} ±0.005
Dhangadi (Kailali)	4.04^{g} ±0.90	nd	0.05^{a}±0.04	0.36^{c}±.09	0.15^{b}±0.04	0.82^{c}±0.06	0.38^{c}±0.0	0.38^{c}±0.00
Mahendranagar (Kanchanpur)	0.66^{bc} ±0.33	0.40^{b}±0.027	0.05^{a}±0.01	0.04^{a}±0.00	0.022^{a}±0.00	0.042^{a}±0.0	0.39^{c}±0.0	0.39^{c}±0.01
Mean	1.77± 0.32	0.33±0.10	0.08±0.03	0.32 ±0.01	0.116±0.02	0.78±0.077	0.31±0.00	0.27±0.00

nd- not detected

Table 5.17 Phytochemical constituents of *Centella asiatica* under different habitats. For each chemical constituent significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA). (N=216).

Chemical constituents	Partiallly shaded grassland	Open grassland	Open agricultural land	Mean	F value	P value
Asiaticoside	1.41 [a] ±1.1	1.71 [b] ±1.033	1.91 [b] ±1.03	1.68±1.6	10.334	0.03
Asiatic acid	0.08 [a] ±0.015	0.07 [a] ±0.018	0.13 [b] ±0.106	0.09±0.016	0.604	0.12
Chicoric acid	0.051 [a] ±0.01	0.058 [a] ±0.01	0.056 [a] ±0.01	0.055±.10	0.603	0.321
Chlorogenic acid	0.24 [a] ±0.18	0.23 [a] ±0.12	0.26 [a] ±0.08	0.25±0.13	0.117	0.190
Quercetin	0.35 [a] ±0.10	0.38 [b] ±0.02	0.38 [b] ±0.002	0.37±0.06	1.154	0.198
Quercetin -3-O – glucuronide	0.17 [a] ±0.004	0.28 [b] ±0.010	0.35 [c] ±0.005	0.247 ± 0.006	17.24	0.025
Kaempferol	0.034 [a] ±0.105	0.35 [a] ±0.10	0.39 [b] ±0.06	0.36±0.09	7.301	0.042
Rosmarinic acid	0.107 [a] ±0.07	0.157 [a] ±0.08	0.183 [b] ±0.17	0.15±0.12	1.301	0.032

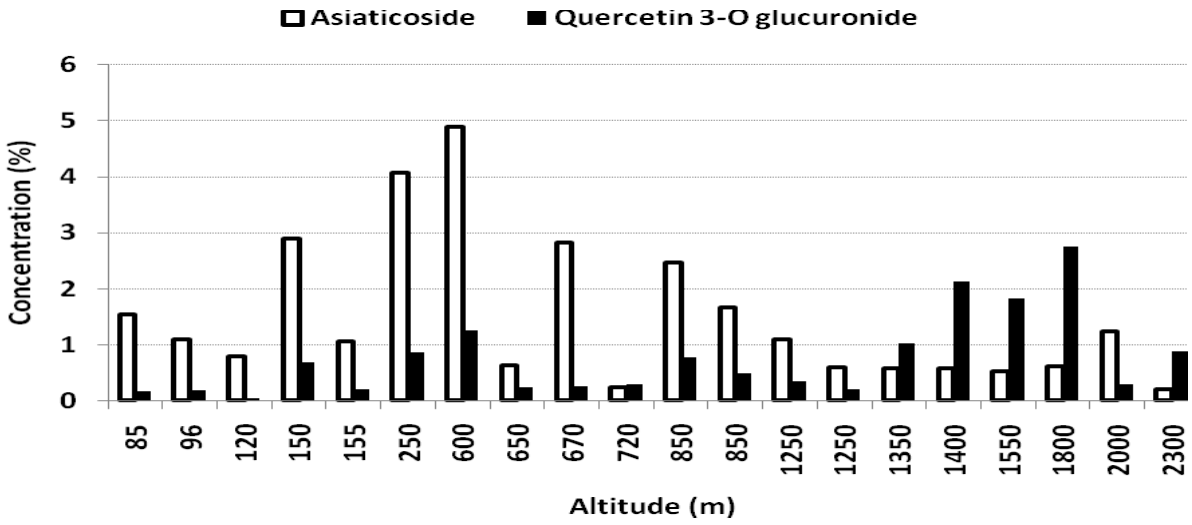


Fig. 5.17 Concentration of asiaticoside and quercetin-3-O-glucuronide in wild samples of C*entella asiatica* collected from various altitudes.

Table 5.18 Concentration (%, d.w.) of various phytochemical constituents of *Centella asiatica* across three ecoregions of Nepal. For each chemical constituent significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA). All samples were collected from wild. Sample size, N=216.

Chemical Constituents	Ecoregion	Mean (± SD)	Minimum	Maximum	F value	P value
Asiaticoside	Eastern	1.20 ± 0.195	1.06	1.53	4.66	0.039
	Central	1.88 ± 0.965	0.24	8.13		
	Western	1.30 ± 0.859	0.59	2.99		
	Average	1.46 ± 1.006	0.63	4.21		
Asiatic acid	Eastern	0.309 ± 0.000	0.31	0.31	0.219	0.804
	Central	0.381 ± 0.202	0.03	0.69		
	Western	0.385 ± 0.025	0.36	0.40		
	Average	0.345 ± 0.075	0.23	0.467		
Chicoric acid	Eastern	0.045 ± 0.005	0.04	0.05	0.971	0.385
	Central	0.095 ± 0.012	0.04	0.09		
	Western	0.098 ± 0.141	0.04	0.50		
	Average	0.066 ± 0.052	0.04	0.213		
Chlorogenic acid	Eastern	0.082 ± 0.047	0.05	0.14	3.563	0.034
	Central	0.520 ± 0.654	0.06	2.46		
	Western	0.261 ± 0.175	0.12	0.72		
	Average	0.287 ± 0.292	0.076	1.106		
Quercetin	Eastern	0.378 ± 0.009	0.37	0.39	0.456	0.636
	Central	0.386 ± 0.019	0.32	0.40		
	Western	0.384 ± 0.016	0.37	0.42		
	Average	0.379 ± 0.014	0.353	0.403		
Quercetin -3- O-glucuronide	Eastern	0.244 ± 0.104	0.09	0.34	20.89	<0.001
	Central	1.328 ± 0.882	0.23	2.79		
	Western	0.512 ± 0.217	0.09	0.85		
	Average	0.694 ± 0.401	0.136	1.32		
Kaempferol	Eastern	0.294 ± 0.171	0.04	0.39	4.17	0.02
	Central	0.370 ± 0.022	0.32	0.40		
	Western	0.372 ± 0.036	0.30	0.41		
	Average	0.345 ± 0.076	0.22	0.4		
Rosmarinic acid	Eastern	0.054 ± 0.003	0.05	0.06	7.42	0.001
	Central	0.160 ± 0.068	0.03	0.34		
	Western	0.120 ± 0.082	0.05	0.28		
	Average	0.113 ± 0.051	0.043	0.226		

5.10.3 Phytochemical constituents of *Centella asiatica* in experimental plants in different environmental condition

Some experimental pots were also prepared with Kathmandu (Kirtipur) germplasm, in order to study the influence of differing environmental conditions on the active constituents of *C. asiatica* (Table 5.20). There was no significant difference in kaemferol among the water level treatments.There was significant difference in quercetin -3-O- glucuronide among the water level treatments , with highest amount was in 70% field water capacity (Table 5.20 A). Highest amount of asiaticoside (0.49 %) was measured in 30% field water capacity. Chicoric acid, rosmarinic acid, kaempferol and quercetin were detected in small amount in all samples.

Significant effect of soil texture on chemical constituents was observed. All analyzed chemical components varied significantly (p<0.001) among the treatments (Table 5.20, B). Highest amount of all chemical constituents was found in treatment with 100% sand. Chicoric acid was detected in small amount in all samples.

Significant difference was obseved in asiaticoside, asiatic acid, chlorogenic acid and quercetin -3 - O- glucuronide concentration among different level of the shade. Highest amount of asiaticoside (0.25%) was measured in full sunlight while least amount in 30% shading (Table 5.20, C). Highest amount of asiatic acid (1.2%) was measured in 70 % shading. Quercetin -3-O- glucuronide was relatively higher in full sunlight than in others. Quercetin could not be detected in any sample analysed during the experiment.

In intergrated manuring experimental plants only four chemical constituents, i.e asiaticoside, asiatic acid, chlorognic acid and quercetin - 3 -O- glucuronide could be detected in all samples (Table 5.20, D). There was no regular pattern of variation in chemical constituents among the treatments. Highest amount of asiaticoside and quercetin -3-O- glucuronide were observed in T_1 (i.e application of inorganic fertilizer only) which were 2.34 and 1.28 times respectively higher than in control. Chemical constituents like chicoric acid, quercetin, kaempferol and rosmarinic acid could not be detected in any sample analysed during the experiment**.**

Table 5.19 Concentration (% d.w.) of selected phytoconstituents from transplanted *Centella asiatica* (sample size(N) =216); nd: not determined. For each chemical constituent significant difference between mean among the ecozone are indicated by different letters (Duncan homogeneity test, α = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Constituents	Ecozone	Mean	Minimum	Maximum	F value	P value
Asiaticoside	Eastern	0.688 ± 0.40	0.16	1.26	0.04	0.96
	Central	0.681 ± 0.61	0.09	1.91		
	Western	0.737 ± 0.48	0.12	1.35		
	Average	0.702 ± 0.496	0.123	1.506		
Asiatic acid	Eastern	0.534 ± 0.35	0.13	1.14	0.133	0.876
	Central	0.60 ± 0.36	0.04	1.11		
	Western	0.607 ± 0.33	0.01	0.99		
	Average	0.58 ± 0.346	0.06	1.08		
Chicoric acid	Eastern	0.0049 ± 0.001	00	0.01	1.131	0.399
	Central	0.023 ± 0.024	0.01	0.04		
	Western	nd	nd	nd		
	Average	0.009 ± 0.008	0.003	0.016		
Chlorogenic acid	Eastern	0.039 ± 0.013	0.02	0.07	4.14	0.028
	Central	0.063 ± 0.02	0.04	0.12		
	Western	0.057 ± 0.01	0.04	0.07		
	Average	0.053 ± 0.014	0.034	0.087		
Quercetin	Eastern	0.023 ± 0.002	0.02	0.03	25.56	<0.001
	Central	0.057 ± 0.00	0.06	0.06		
	Western	0.228 ± 0.081	0.08	0.40		
	Average	0.102 ± 0.027	0.053	0.163		
Quercetin -3-O-glucuronide	Eastern	0.214 ± 0.06	0.08	0.28	2.786	0.106
	Central	0.288 ± 0.118	0.08	0.51		
	Western	nd	nd	nd		
	Average	0.167 ± 0.059	0.053	0.263		
Kaempferol	Eastern	nd	nd	nd		
	Central	nd	nd	nd		
	Western	nd	nd	nd		
	Average	nd	nd	nd		
Rosmarinic acid	Eastern	nd	nd	nd		
	Central	nd	nd	nd		
	Western	nd	nd	nd		
	Average	nd	nd	nd		

Table 5.20. Phytochemical constituents of *Centella asiatica* in experimental plants in differing environmental conditions (N= 100). For each chemical constituent significant difference between mean among the treatments are indicated by different letters (Duncan homogeneity test, α = 0.05).

A. Effects of moisture levels (sample size (N) = 20).

Treatment	Amount (%) Kaempferol	Chicoric acid (%)	Quercetin- 3-O-glucuronide (%)	Quercetin (%)	Asiaticoside (%)	Asiatic acid (%)	Rosmarinic Acid (%)	Chlorogenic Acid (%)
125 % FWC	0.01 [a]	0.005 [a]	0.274 [b]	0.033[b]	0.12 [a]	0.007[a]	0.005[a]	0.03 [a]
100% FWC	0.014 [a]	0.004 [a]	0.068 [a]	0.021[a]	0.16 [b]	0.93 [b]	0.009[a]	0.036 [a]
70% FWC	0.011 [a]	nd	0.31 [c]	0.031[b]	0.213 [c]	0.001 [a]	0.005 [a]	0.07 [b]
30 % FWC	0.011 [a]	nd	0.27 [b]	0.037 [b]	0.493[c]	0.002 [a]	0.018 [b]	0.037°

B.Effect of soil composition (sample size (N) = 30).

Treatment	Amount (%) Kaempferol	Chicoric acid (%)	Quercetin- 3-O-glucuronide (%)	Quercetin (%)	Asiaticoside (%)	Asiatic acid (%)	Rosmarinic Acid (%)	Chlorogenic Acid(%)
0% sand	0.013 [a]	0.002 [a]	0.103 [b]	0.029[b]	0.22 [b]	0.49[d]	0.018 [bc]	0.06 [f]
20% sand	0.019 [b]	<0.001	0.091 [a]	0.104[d]	0.27 [c]	0.60 [e]	0.013 [a]	0.035 [d]
40%sand	0.012 [a]	<0.001	0.120 [c]	0.02 [a]	0.102[a]	0.10 [c]	0.021 [d]	0.004°
60%sand	0.011 [a]	0.027	0.148 [d]	0.023 [a]	0.122[a]	0.075[b]	0.016 [b]	0.007 [b]
80% sand	0.013 [a]	<0.001[a]	0.204 [e]	0.021[a]	0.13[a]	0.045[a]	0.020 [cd]	0.023 [c]
100% sand	0.027 [c]	<0.001[a]	0.274 [f]	0.047[c]	0.56 [d]	0.72 [f]	0.062 [e]	0.38 [e]

C. Effects of shading (sample size (N) =20).

Treatments	Amount (%) Kaempferol	Chicoric acid (%)	Quercetin- 3-O-glucuronide (%)	Quercetin (%)	Asiaticoside (%)	Asiatic acid (%)	Rosmarinic Acid (%)	Chlorogenic Acid(%)
30 % shade	0.003 [a]	001 [a]	0.167 [c]	nd	0.158[a]	1.19 [b]	0.009	0.001 [a]
50 % shade	0.009 [b]	0.003 [a]	0.103 [b]	nd	0.176[b]	0.009 [a]	0.004 [a]	0.053[b]
70 % shade	0.007 [ab]	0.028[b]	0.095 [b]	nd	0.18 [b]	0.009[a]	0.014 [a]	0.005 [a]
0 % shade	0.007 [ab]	0.003[a]	0.022 [a]	nd	0.25 [c]	0.043[a]	0.007 [a]	0.027 [b]

D. Effect of Integrated manuring (Sample size (N) = 30.)

Treatment	Kaempferol	Chicoric acid (%)	Quercetin- 3-O-glucuronide (%)	Quercetin (%)	Asiaticoside (%)	Asiatic acid (%)	Rosmarinic Acid (%)	Chlorogenic Acid(%)
100% Urea	nd	<0.001[a]	0.27[b]	nd	0.22[a]	<0.001	nd	0.008 [a] ±0.00
75% Urea: 25% FYM	nd	<0.001[a]	0.26[b]	nd	0.095	0.001[a]	nd	0.003 [a] ± 0.00
50% Urea: 50% FYM	nd	0.008[a]	0.157[a]	nd	0.115	0.001[a]	nd	0.106 [b] ±0.01
25% Urea : 75% FYM	nd	0.153[b]	0.19[a]	nd	0.185[c]	0.048 [b]	nd	0.094 [b] ±0.01
100 % FYM	nd	0.002[a]	0.23[ab]	nd	0.157[c]	0.02[b]	nd	0.010 [a] ±0.00
Control	nd	<0.001[a]	0.21[ab]	nd	0.094[b]	0.004[a]	nd	0.081 [b] ±0.01

5.11 Essential Oil

Fifty two compounds of essential oil was recorded from analysis of all samples in Nepal (Table 5.22). The yield of essential oil across the habiats ranged from 0.09 to 0.12% (shady grassland = 0.12%, open grassland = 0.1%, open agricultural land = 0.09%) (Table 5.21). Over 94% of the constituents of the *C. asiatica* essential oil were determined in the collected samples, and the number of identified compounds were different depending on the habitat: 39 in the essential oil from plants grown in shady grassland, 34 in open grassland, and 36 in open agricultural land (Table 5.21). GC/FID analysis showed that the oils were characterized by a high amount of sesquiterpenoid hydrocarbons ranging from 70.25 to 82.09%, mainly γ-caryophyllene (9.24-32.3%), β-caryophyllene (7.5–24.2%), β-farnesene (1.7–18.89%), α-humulene (0.05–17.09%) (Table 5.22). The oxygenated sesquiterpenoid fraction (3.75-10.53%) was mainly composed of caryophyllene oxide (0.56-8.46%); whereas the monoterpenoid fraction was very low in all three oil samples (0.50-2.34%).

Among all the compounds, γ-caryophyllene, β-caryophyllene and caryophyllene oxide were present in higher amounts in plants grown in open agricultural land, whereas α-humulene and β-farnesene in plants grown in open grassland.

Table 5.21 Yield of essential oil and soil characters of habitat relative to the analyzed samples of *Centella asiatica.*

Attributes	Partially shaded grassland (Under shrub)	Open Grassland	Open agricultural land
Soil pH	6.01	5.81	5.71
Soil nitrogen (%)	0.28	0.16	0.18
Soil Organic carbon (%)	3.8	1.28	2.25
Soil Organic matter (%)	6.58	2.21	3.89
Oil yield (%)	0.12	0.10	0.09
Oil colour	Pale yellow	Pale yellow	Pale yellow
No of identified chemical components	39	34	36

Table 5.22 Chemical composition (%) of *Centella asiatica* essential oils from different habitats.

SN	Compound [a]	Kovat Index [b]	Shady grassland [c]	Open grassland [c]	Open land[c]	Agricultural
1	Thujopsene	926	-	0.34	0.48	
2	α-Thujene	931	0.64	-	1.12	
3	α-Pinene	939	0.02	0.06	-	
4	Camphene	953	-	-	0.05	
5	β-Pinene	980	-	0.02	0.42	
6	3,6-Heptadiene-2-ol	995	0.15	-	-	
7	β-Cymene	1030	-	0.08	0.27	
8	Eucalyptol	1033	1.57	-	-	
9	3-Nonen-2-one	1065	0.49	0.06	0.80	
10	β-Linalool	1098	0.13	1.36	-	
11	L-Camphor	1143	-	-	0.59	
12	trans-Borneol	1169	0.11	-	0.08	
13	4 –Terpinenol	1177	0.18	-	0.11	
14	α –Terpeneol	1189	0.12	-	0.07	
15	Cis-Geraniol	1255	-	0.98	1.24	
16	Isobornyl acetate	1286	0.06	-	-	
17	4,8a-Dimethyloctahydro-4a(2H)-naphthalenol	1373	-	5.24	0.23	
18	α –Copaene	1377	0.54	0.37	0.39	
19	7-Tetradecene	1378	-	0.08	-	
20	β-Cubebene	1390	-	1.24	0.79	
21	β-Elemene	1391	5.01	3.93	2.34	
22	γ- Caryophyllene	1420	26.45	9.24	32.30	
23	β-Caryophyllene	1428	24.20	7.50	24.50	
24	β-Gurjunene	1433	0.55	0.13	0.20	
25	γ-Elemene	1437	1.05	-	0.81	
26	Isocaryophyllene	1438	3.01	5.24	-	
27	Aromadendrene	1441	0.78	0.43	-	
28	Cis-β-Guainene	1440	-	-	0.58	
29	α –Humulene	1455	0.05	17.09	6.86	
30	β-Farnesene	1456	12.36	18.89	1.70	
31	Alloromadendrine	1460	-	0.73	0.55	
32	β-Acoradiene	1470	0.34	0.09	-	

Contd…. Table 5.22

33	γ-Murrocene	1476	-	-	0.15
34	Germacrene-D	1485	1.82	2.5	0.08
35	β-Selinene	1490	3.8	-	-
36	α-Selinene	1493	0.75	-	-
37	α-Chamigrene	1499	0.98	0.60	1.09
38	γ-Cadinene	1514	0.17	-	-
39	β –Candinene	1519	0.17	2.13	0.37
40	α-Panasinsen	1535	0.06	0.06	0.75
41	Selina-3,7(11)-diene	1543	1.22	-	-
42	Caryophyllene oxide	1581	0.56	7.68	8.46
43	-(-)Spathulenol	1578	0.47	0.80	0.41
44	Viridiflorol	1593	-	0.56	0.81
45	Valeranone	1675	1.20	0.03	-
46	Isoaromadendrene epoxide	1740	0.20	1.46	-
47	Aristolene epoxide	1763	0.83	-	0.75
48	1-Naphthalenol	2248	0.30	2.7	1.35
49	3,7,11,15-Tetramethyl α-hexadecane-1-ol	2282	1.18	0.06	0.88
50	1R,4s,7s,11R-2,2,4,8-Tetramethytricyclone		1.33	0.51	1.23
51	1H-Cyclopropa[a]naphthalene, decahydro-1		1.64	0.95	3.08
52	Tricyclo[5.2.2.0(1,6)undecan-3-ol,2-me		1.34	0.90	0.29
	Total		94.70	94.01	95.98

[a] Compounds are listed in order of elution time on DB-5 column.

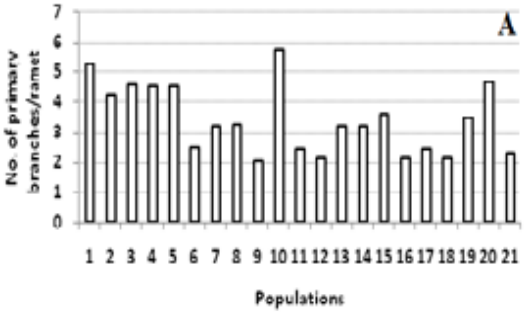
[b] Retention indices calculated on DB-5 capillary column.

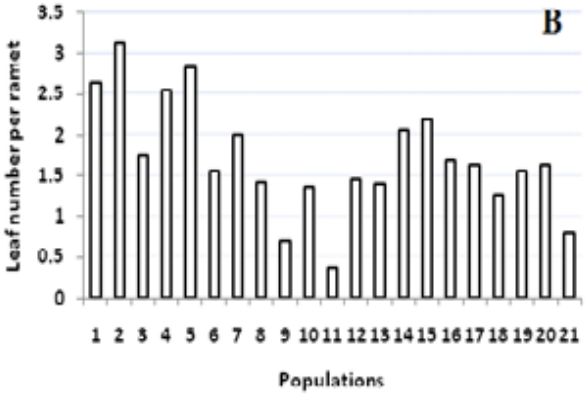
[c] Relative area percentage: peak area relative to total peak area percent,calculated on DB-5 column by GC-FID analysis.

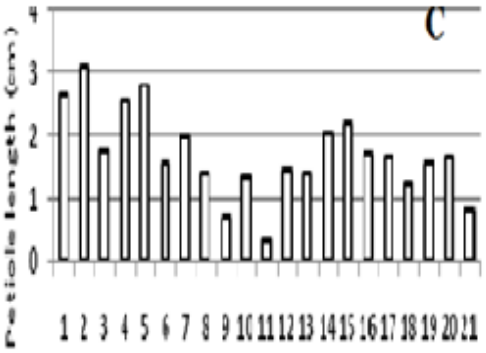
5.12 Genetic Diversity

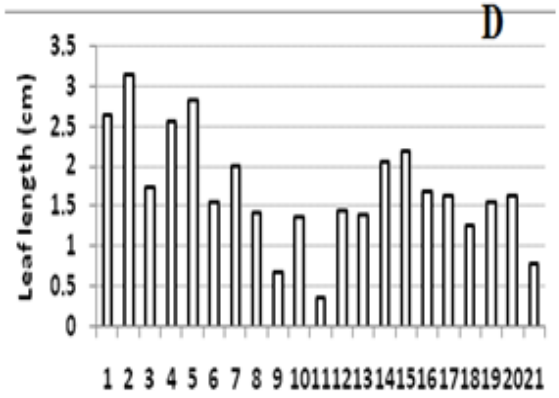
5.12.1 Morphological Analysis

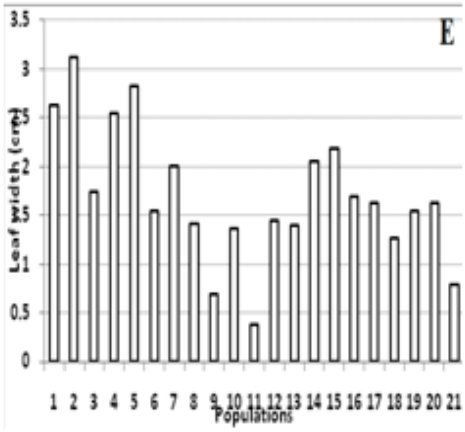
Several morphological and physiological characters were measured from transplanted samples to understand genetic diversity in different population of *Centella asiatica.* Variance analysis showed that all evaluated traits were significantly different (Fig 5.18 and Appendix 1). Mean, minimum and maximum values of each trait have been represented in Table 5.23. High coefficients of variability were obtained for number of flowers per ramet (49.8%), specific leaf area (38.04%) and seed mass (37.03%). Mean comparisons based on Duncan test showed that some of the populations v*iz*. CABar, CAChit, CADhan, CAKan and CALam were superior to others with regard to number of primary branches, plant dry mass and leaf number per plant. Plant biomass was significantly correlated with that of leaf length (0.68), leaf width (0.67, leaf number (0.69) and flower number (0.80) (Table 5.24). Dendrogram based on morphological data was drawn to display the phenetic relationships among different populations of *Centella asiatica*. All populations were represented into two major groups (Fig 5.19, Plate 2). Cluster analysis indicated that populations CAKir, CAMa, CAGor, CADam, CASur, CADham, CAPok, CALal and CADhan, CAI and CAPyu of group I had relatively small leaves, with dentate to serrate leaf margin, high numbers of flower per ramet, with low biomass production and high seed production . In contrast, populations such as CASun, CAJha, CA,Mak, CADan, CABan, CABar, CAChit, CADhan, CAKan and CALam of group II were generally erect, characterized by large leaves on long petioles, crenate to entire leaf margin, more branches and fast growing, high biomass production and less number of flowers and seeds per ramet.

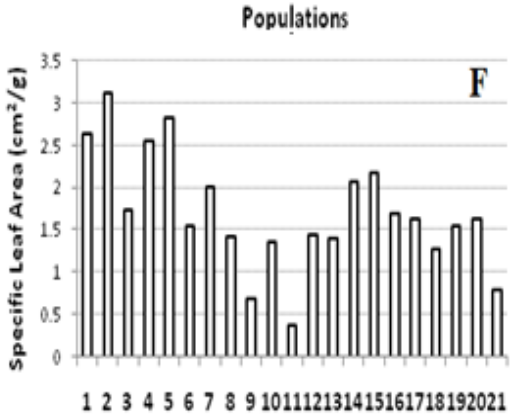

A
No. of primary branches/ramet
Populations


B
Leaf number per ramet
Populations


C
Petiole length (cm)
Populations


D
Leaf length (cm)
Populations


E
Leaf width (cm)
Populations


F
Specific Leaf Area (cm²/g)
Populations

Contd….

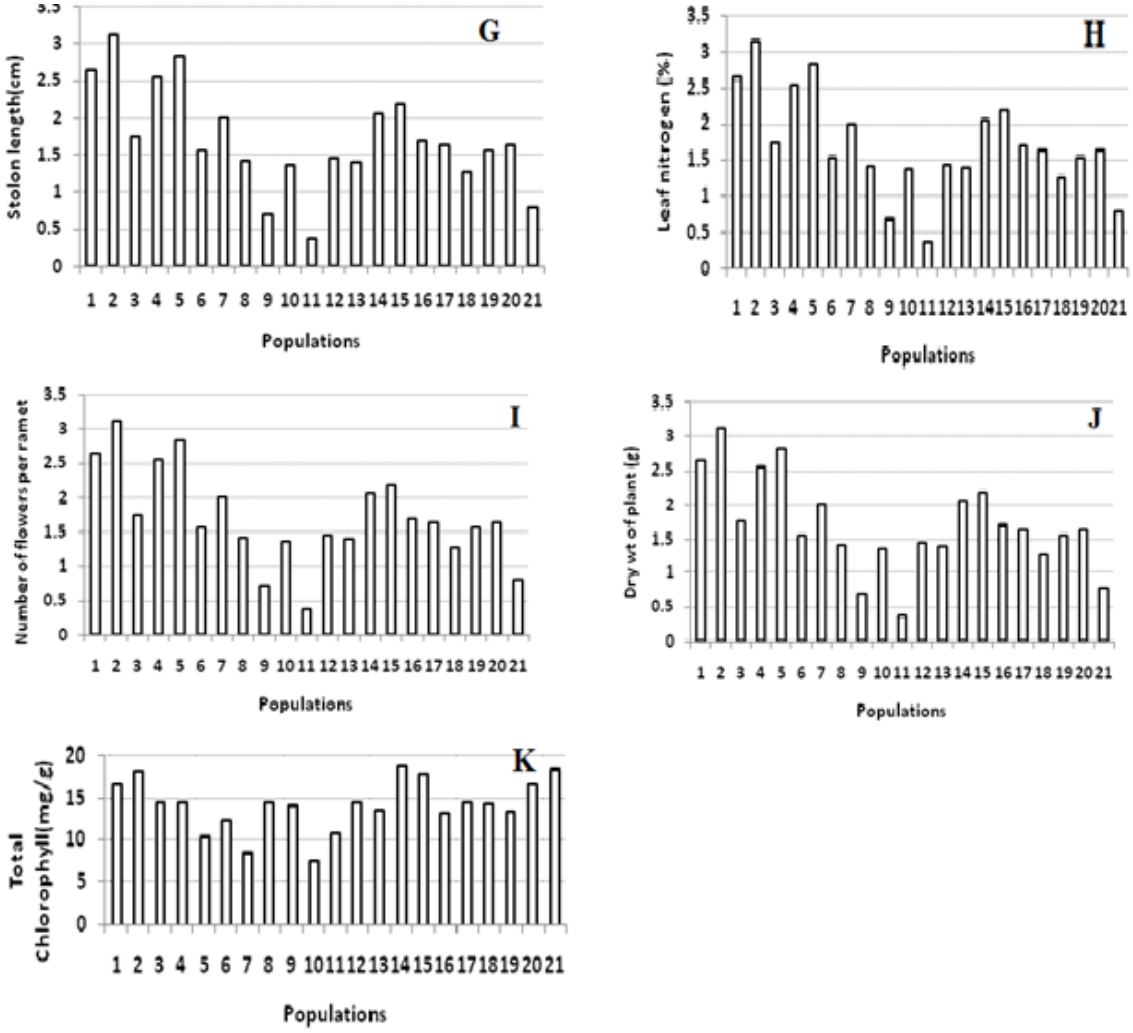


Fig 5.18 Growth traits of 21 populations of *Centella asiatica*; A-Number of primary branches per ramet, B-Number of leaves per ramet, C- Petiole length, D- Leaf length, E-Leaf width ,F- Specific Leaf Area, G- Stolon length, H- Leaf nitrogen, I- Number of Flowers per ramet,, J- Dry wt. of individual ramet, K-Total chlorophyll content in leaves; Populations 1-Ilam, 2-Jhapa, 3-Sunsari, 4-Hetauda, 5-Bardiya, 6-Lalitpur, 7-Kirtipur, 8-Matatirtha, 9-Gorkha,10- Lamjung, 11-Dhampus, 12-Pokhara, 13-Pyuthan, 14-Dang, 15- Chitwan, 16- Banke, 17-Kailali, 18-Dhankuta , 19-Surkhet, 20- Mahendranagar, 21-Daman

Plate 2. **Two different morphotypes of *Centella asiatica*.A,B-Tall and more branching type with crenate to entire leaf margin; C,D Short and less branching type with dentate –serrate leaf margin.**

Table 5.23. Mean, maximum, minimum and coefficient of variation (CV %) of different traits, studied among 21 populations (transplanted samples) of *Centella asiatica.*

Attributes	Sample size	Mean ± SD	CV	Min	Max
Petiole length (cm)	1890	8.6 ± 1.91	22.2	2.21	15.15
Leaf length (cm)	1890	1.81 ± 0.4	22.09	1.26	2.45
Leaf width (cm)	1890	3.05 ± 0.62	20.32	2.26	4.26
SLA(cm^2/g)	1890	347 ± 132	38.04	166.43	924.37
Leaf nitrogen (%)	420	2.22 ± 0.39	17.56	1.85	3.27
Stolon length (cm)	630	8.01 ± 2.32	28.96	4.64	10.56
Leaf number/ramet	630	3.73 ± 1.35	36.19	2.32	5.36
Flower number/ramet	630	7.55 ± 3.76	49.80	0	24
Seeds/ramet	630	12.51 ± 1.21	9.67	0	18
Seed mass (g) (100 seeds)	1600	0.108 ± 0.04	37.03	0.098	14.3
Dry mass of individual plant (g)	630	1.53 ± 0.54	35.29	0.38	3.27

Table 5.24 Spearman's correlation coefficient among morphological and physiological traits of *C. asiatica*.

Traits	Petiole length	Leaf length	Leaf width	SLA	Leaf nitrogen	Stolon length	Leaf number	Flower number
Leaf length	0.29**							
Leaf width	0.26**	0.66**						
SLA	ns	0.58**	0.64 **					
Leaf nitrogen	0.17**	ns	0.08*	0.15*				
Stolon length	ns	ns	ns	ns	–0.22**			
Leaf number	0.11**	0.16**	ns	ns	ns	–0.1**		
Flower number	0.22**	ns	ns	0.02*	ns	ns	ns	
Dry mass of plant	0.54**	0.68**	0.67**	0.18**	0.15**	0.61**	0.69**	0.80**

** Correlation is significant at 0.01 level (2- tailed) *Correlation is significant at 0.05 level (2-tailed); ns: non significant

5.12.2 Random Amplified Polymorphic DNAs (RAPD) analysis

Analysis of 21 populations of *Centella asiatica* revealed 81% polymorphism using eight primers (Plate 3). Out of 21 tested primers, only eight primers produced scorable, reproducible bands; therefore only eight primers were used for analysis. The numbers of scorable polymorphic markers generated were 35 out of 42 total markers (Table 5.25). The primer with maximum number of polymorphic bands was OPC-13 (8 bands) and the minimum was OPC-3 (3 bands). No primer exhibited complete monomorphism. Hence the range of the bands generated for all the primers fell between 3 and 8. The average number of polymorphic bands per primer generated was 4.37 out of the total number of bands (5.25). The primers OPA-01, OPC-10, OPC-13 and OPC-18 exhibited 100% polymorphism with all the populations. The similarity

matrix is represented in Table 5.26. The lowest similarity (0.52) was between the population number 10 from Daman and number 19 from Kanchanpur. The highest similarity (0.92) was observed between the population numbers 11 and 12 from Kirtipur and Matatirtha. The cophenetic correlation coefficient indicated a strong correlation (r = 0.95) between the similarity matrix and the UPGMA dendrogram, indicating a remarkable relationships among the populations. According to the dendrogram (Fig 5.20), at a similarity level of 0.61-0.65 the populations were divided into two main groups and one outlier. Group I involved eight populations from different regions of low land site while group II involved twelve populations. Population from Lamjung district was quite distinct from other groups in dendrogram. In RAPD-based clustering, all populations that belonged to areas in close vicinity formed close groups, for example Kirtipur and Matatirtha; Dhampus and Pokhara; and Banke and Brdiya.

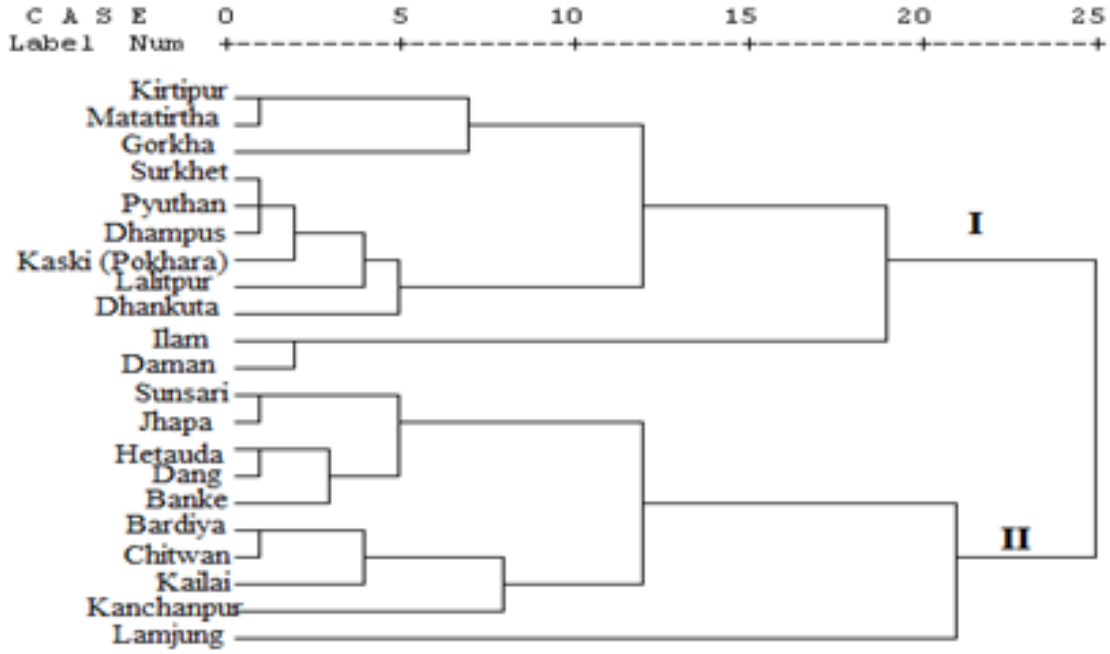


Fig 5.19. Dendrogram showing the phenetic relationships among 21 populations of *Centella asiatica* based on hierarchial clustering from morphological data matrix.

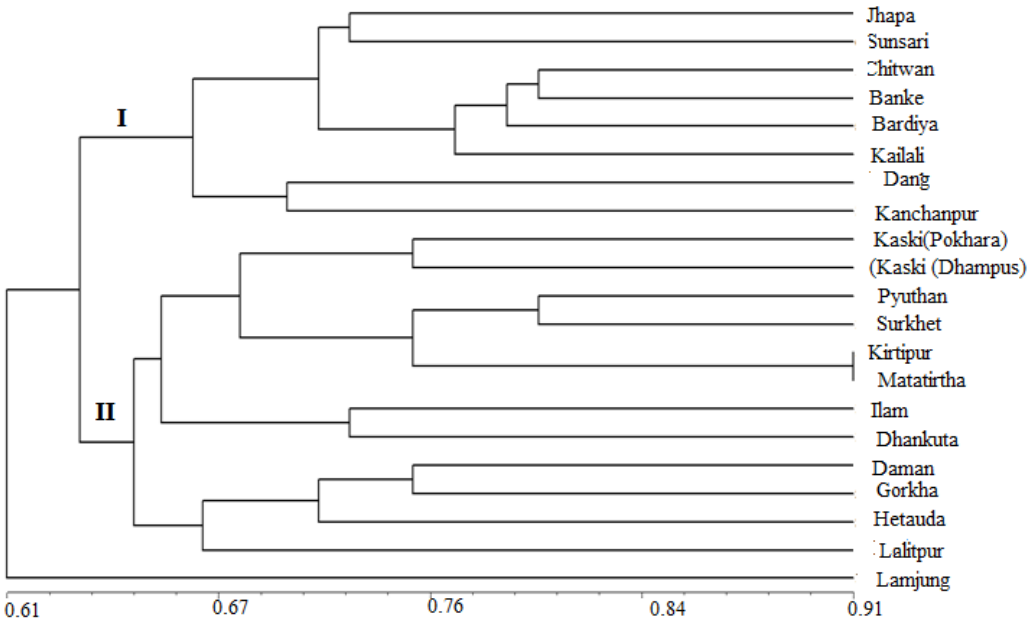
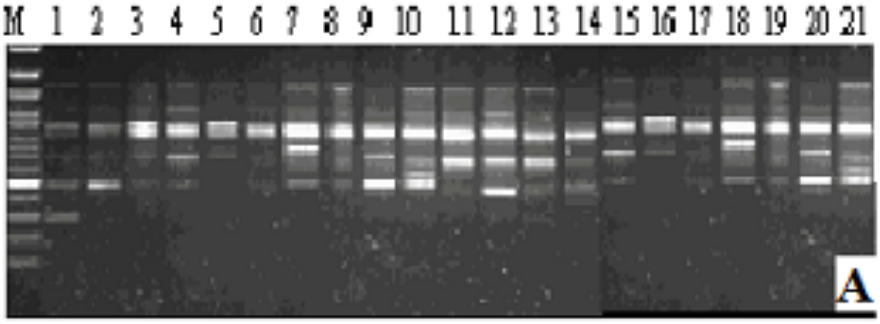
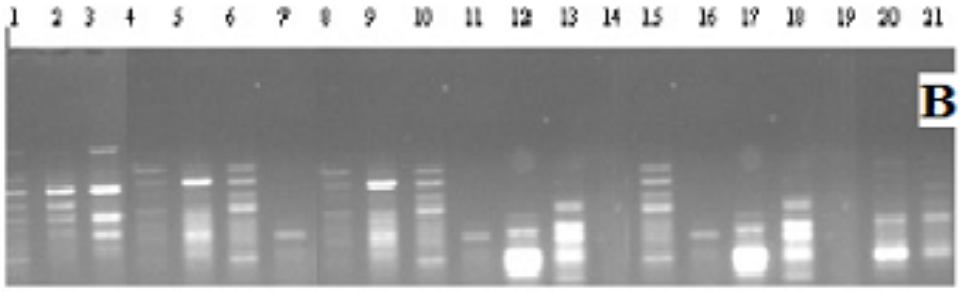


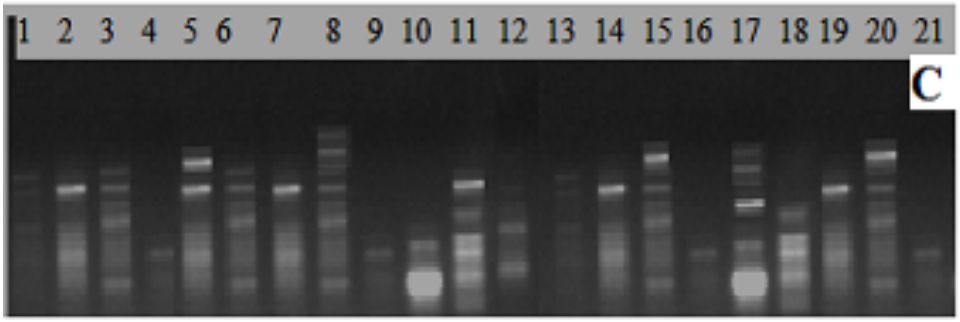
Fig 5.20 UPGMA Dendrogram for 21 populations of *Centella asiatica* by RAPD analysis using Jaccard's similarity coefficient.

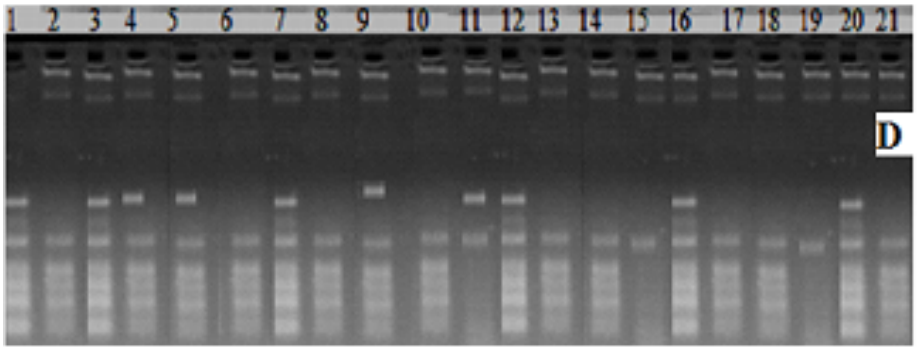
Table 5.25 RAPD analysis in percentage of polymorphism, total number of bands, and polymorphic band.

Primer code	Primer sequence	Total bands	Polymorphic bands	Polymorphism (%)
OPA -01	CAG GCC CTT C	5	5	100
OPA-03	AGT CAG CCA C	6	5	83.33
OPA-05	AGG GGT CTT G	7	5	71.42
OPC-03	GGG GGT CTT T	3	2	66.67
OPC-04	CCG CAT CTA C	4	1	25
OPC-10	TGT CTG GGT G	5	5	100
OPC-13	AAG CCT CGT C	8	8	100
OPC-18	TGA GTG GGT G	4	4	100
Total		42	35	646.42
Mean		5.25	4.37	80.80


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
C


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
D

Contd……

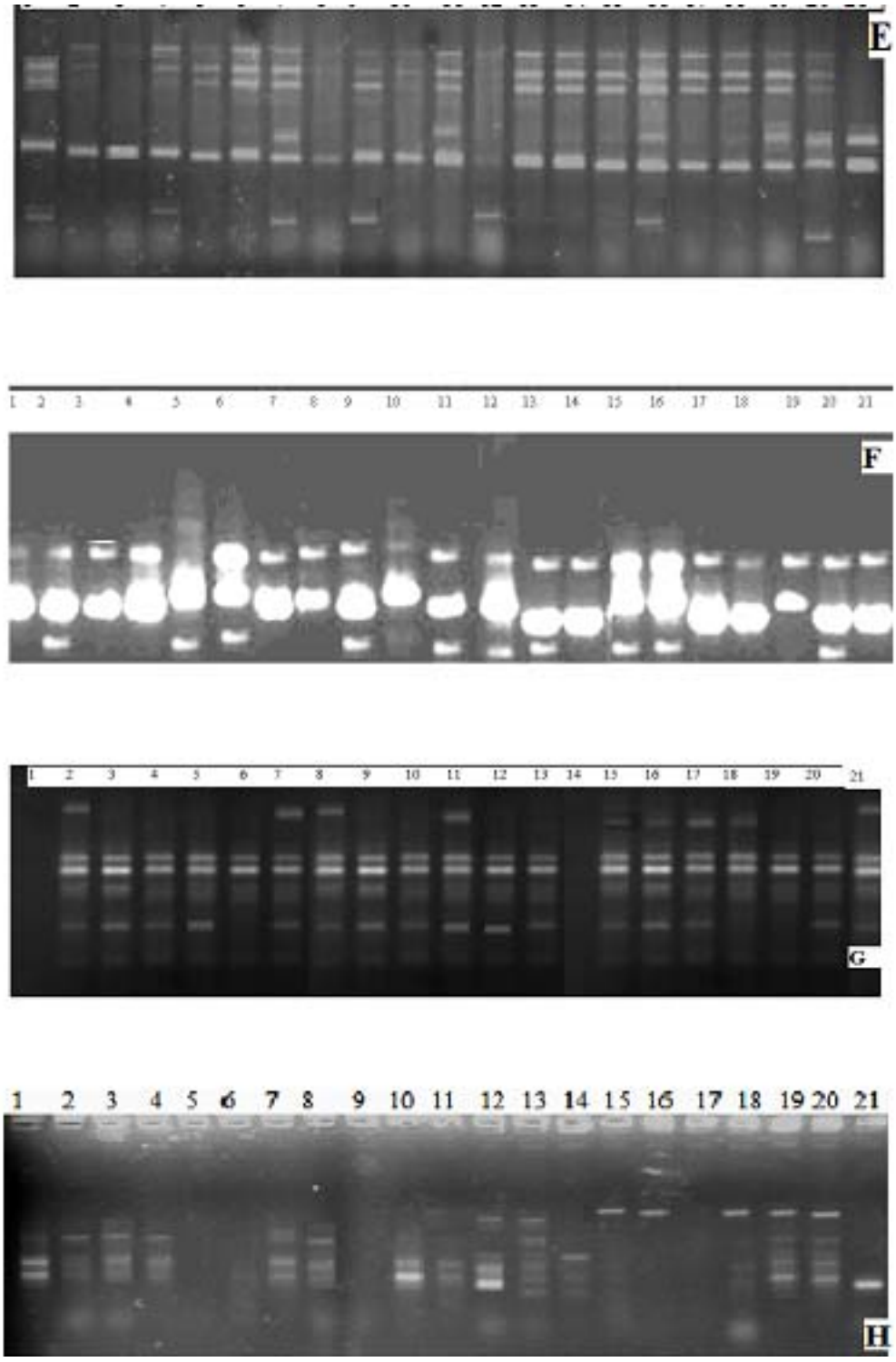


Plate 3. **RAPD profile of *Centella asiatica* using primers A. OPA-5, B. OPC-13, C. OPA -1 , D. OPC-4, E. OPA- 3, F. OPC -3, G. OPC-10, H. OPC-18 Electrophoresis performed on 1.5% agarose gel. M: Molecular Marker. Lanes 1-21: DNA isoalted from 21 different poulations of *C. asiatica.***

Table 5. 26 Jaccards' Similarity index of RAPD banding patterns among 21 *Centella asiatica* populations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Jhapa (1)	1																				
Chitwan (2)	0.778	1																			
Banke (3)	0.756	0.8	1																		
Kailai (4)	0.688	0.733	0.8	1																	
Bardiya (5)	0.644	0.778	0.8	0.778	1																
Sunsari (6)	0.733	0.733	0.71	0.733	0.733	1															
Kasi, Pok (7)	0.556	0.733	0.66	0.6889	0.688	0.555	1														
Kaski, Dhampus (8)	0.578	0.666	0.778	0.7556	0.755	0.578	0.756	1													
Pyuthan (9)	0.666	0.711	0.64	0.711	0.711	0.622	0.711	0.733	1												
Daman (10)	0.644	0.6	0.578	0.6	0.6	0.6	0.644	0.711	0.666	1											
Kirtipur (1 1)	0.577	0.666	0.699	0.756	0.755	0.622	0.711	0.778	0.733	0.755	1										
Matatirtha (12)	0. 622	0.666	0.688	0.711	0.711	0.622	0.667	0.688	0.688	0.755	0.911	1									
Ilam (13)	0.533	0.533	0.644	0.711	0.622	0.533	0.622	0.733	0.733	0.622	0.733	0.644	1								
Dhankuta (14)	0.533	0.622	0.733	0.667	0.666	0.488	0.667	0.733	0.644	0.533	0.733	0.644	0.733	1							
Surkhet (15)	0.577	0.622	0.644	0.711	0.667	0.666	0.622	0.733	0.733	0.8	0.777	0.733	0.644	0.688	1						
Dang (16)	0.688	0.689	0.711	0.689	0.689	0.733	0.688	0.533	0.667	0.6	0.755	0.711	0.622	0.577	0.622	1					
Lalitpur (17)	0.555	0.6	0.622	0.556	0.644	0.56	0.644	0.577	0.667	0.644	0.666	0.667	0.57	0.666	0.622	0.644	1				
Lamjung (18)	0.577	0.622	0.644	0.667	0.578	0.711	0.667	0.556	0.6	0.533	0.6	0.644	0.64	0.55	0.597	0.62	0.667	1			
Kanchanpur (19)	0.622	0.711	0.644	0.667	0.711	0.577	0.711	0.6	0.6	0.52	0.688	0.644	0.6	0.644	0.511	0.711	0.577	0.6	1		
Gorkha (20)	0.644	0.644	0.578	0.689	0.644	0.644	0.644	0.577	0.755	0.6	0.667	0.667	0.666	0.577	0.622	0.644	0.733	0.6667	0.622	1	
Hetauda (21)	0.644	0.688	0.711	0.689	0.689	0.555	0.5556	0.6667	0.711	0.6	0.711	0.756	0.711	0.711	0.578	0.555	0.644	0.622	0.666	0.733	1

6. DISCUSSION

6.1 Distribution

Centella asiatica has wide distribution in terms of elevation (<85 to about 3300 m asl), habitat types (open moist land, agricultural land, grassland to shady fallow land) and physiography (eastern to western Nepal, terai plain to midhills of Mahabharat range) in Nepal (Tables 3.1, Fig 5.1 & Appendix 2). It was also reported from wetland system of terai region (Burlakoti and Karmacharya 2004). This species has been reported from shady marshy ground and roadside ditches of Sikkim and Bhutan from altitudinal range of 400 to 1500 masl (Grierson and Long 1999). It appears that 'open moist land' is the most common habitat of *C. asiatica* because majority of the specimens were collected from such habitat .The fallow land was regularly disturbed during plantation of paddy. In shady grassland, density of *C. asiatica* was higher than in nearby open grass land and open agricultural land (Table 5.1). It appears that deep shade within dense grassland was not a suitable microhabitat for *C. asiatica* as only 1.31% specimens were collected from forest gap. Thus distribution of this plant appeared to be determined by light availability of microhabitat. Majority of the specimens were collected from tropical (50%), and subtropical (38%) belts and least number of specimens from temperate belts, indicated that *C. asiatica* is more common in warm tropical to subtropical climate.

6.2 Abundance

Population density and above ground biomass varied with habitats (Table 5.2). Highest density (103.24 pl/m^2) and biomass yield (54.50 g/m^2) was found in relatively undisturbed moist partially shaded grassland. This could possibly be due to high nutrient content (Table 5.15). Lowest ramet density of *C.asiatica* in agricultural land could be due to periodic disturbance during agricultural practices. Given the highest plant density and biomass production in partially shaded grassland, this habitat can be considered as the most suitable habitat for the growth of this plant under natural conditions. Since there has not been any earlier study on population of this plant, comparable data on population and abundance of this species is not available from Nepal and elsewhere (Devkota and Jha 2008); but experimental shading has shown that plant biomass yield of *C. asiatica* was the highest under the condition of partial shading (Mathur *et al*.2000).

There was only marginal significant relation of density and plant biomass with soil attributes (Appendix 3). The lack of strong relationship between soil variables and abundance of this plant could be due to the overriding effect of other environmental factors such as light, temperature and moisture. Similar situation has been also reported for *Curculigo orchioides* (Shrestha 2010).

Population density and plant biomass of *Centella asiatica* were the highest in eastern Nepal and values of both these parameters declined from eastern Nepal to westward (Fig 5.2). This pattern could be related to east-west moisture gradient due to decreasing pattern of monsoon from east Nepal to west (Singh 1999). Since *C. asiatica* prefers moist habitat, the eastern Nepal with high monsoon rain might be more suitable than west Nepal for growth of this plant.

6.3 Leaf Traits

Leaf area varied from 3.02 to 11.51 cm^2 with average value of 7.26 cm^2. The variation in leaf area among the populations was due to different habitat conditions of the collection site. Comparison between *Centella asiatica* of Nepal with *C. asiatica* from Bahamas and Louisiana studied by Booncong (1989) as shown in Appendix 4, revealed that populations in Nepal have smaller leaf area. However, the leaf area of samples of Nepal match the description of *C. asiatica* of Malaysia (Pick Kiong 2004).

The petiole length of leaves of *Centella asiatica* of Nepal (2.36 to 9.40 cm) was similar to those reported from Malaysia by Pick Kiong (2004), and Thailand and Costa Rica by Booncong (1989). But it was shorter than that of Bahamas and Louisiana which owned a longer petiole at the range of 5 to 25 cm (Booncong 1989). This could be due to the fact stated by Solet *et al.* (1998) that *C. asiatica* grown at sunny places such as Malaysia and Thailand had shorter petiole. Stolon length among the populations of Nepal varied significantly ($p < 0.001$) ranged from 3.15 to 7.28 cm. The value was similar to those reported from Malaysia by Pick Kiong (2004).

6.4 Variation in Growth Characters

Variation in growth characters and reproductive outputs among the individuals of *Centella asiatica* growing in different habitats (open agricultural land, open grass land, and partially shaded grassland) (Table 5.1) represented growth responses to resource

availability. The variation in petiole length was adaptive response to light. Plasticity of petiole helps to place the lamina in areas of high light (deKroon and Hutchings 1995). Herbaceous dicot plants growing in open habitat often respond to shading by elongating internodes and petiole length (Huber 1996), which improves access to light (Falster and Westoby 2003). Long petiole raises the leaf lamina and enables the plant to receive adequate light when density and height of associated species is high. This is a common adaptive strategy of light demanding herbaceous species. Shortest petiole length of *C. asiatica* at agricultural land was due to less density of associated species and more open area as it received sufficient amount of light. There is therefore no need to develop long petiole for the plant. Plasticity in lamina and petiole occurs both between and within plants in response to contrasting exposure to light (Niklas 1999, Niinemets and Fleck 2002). Due to open land both in agricultural as well as grassland, plant easily got sufficient light and there was no need of extension of petiole. This explains the short petiole length in leaf of *C. asiatica* in open habitat.

There was significant difference (p >0.001) in SLA among samples from different habitats. The variation in SLA might be due to different light intensity (Hughes and Cockshull 1972, Nederhoff *et al* 1972, Heuvelink and Marcelis, 1996) or may be due to variation in leaf nutrient. The highest SLA (498.0 cm^2/g) of *C. asiatica* in shaded grass land may be due to reduction in available light to the leaves when the plant density was high. A positive effect of plant density on SLA has been found in other crops, e.g. potato (Vos 1995), tomato (Heuvelink and Marcelis 1996) and *Impatiens capensis* (Maliakal *et al*. 1999). Plants grown in high light generally have thick leaves with low SLA (Bjorkman 1981). Average SLA of *C. asiatica* lies near the median range (67 to 715cm^2/g) of global data set for 2548 species compiled by Wright *et al*. (2004).

There was significant difference in number of flower per ramet among the habitats (Table 5.1). There appears trade off between densities of ramets and number of flowers per inflorescence. The number of flowers per inflorescence was highest at open agricultural land where ramet density was the lowest. Failure of large proportion of ramets to bear flower at shady grassland may be due to density dependent factors such as competition for resources (eg. nitrogen), space as well as light factor. The lowest number of flowers per inflorescence in shady grassland might also be due to light factor. Dense growth of associated species could also be less favourable for the production of flowers. The plants of shady grassland tended to invest fewer resources

in sexual fecundity and more in traits ensuring vegetative offshoots. Differential patterns of ramet recruitment and growth in different habitats also underlie variation in population growth rates in other perennial herbs (Tictin and Nantel 2004).

Variations in leaf form and patterns among populations could be attributed to the environmental influences of microhabitats as shown in *Erythroxylum coca* (Johnson *et al.* 2002). Mathur *et al.* (2000) also reported that 16 acccessions of *C. asiatica* collected from different geographical locations in India were found to harbor wide differences in their morphology. Evolution by means of mutation, segmental duplication and structural alterations of chromosomes played an important role in the morphological variations among the *C. asiatica* accessions (Das and Mallick 1991). Adaptation to different environments causes the numerical and structural alteration of chromosomes in plants genome. The increase or decrease in chromosome numbers might have been due to duplication of chromosomes or translocation between the chromosomes at a very early stage of evolution. An additional chromosome (B chromosome) reported in *C. asiatica* growing in the higher altitude area (1510 to 2136 m), sub temperate and halophytic zones influenced the genomic length volume and asiaticoside content of the plants (Das and Mallick 1991).

6.5 Relative Growth Rate (RGR)

Relative growth rate of *Centella asiatica* of Nepal was 0.64 $g.g^{-1}$ $month^{-1}$ which is lower than in *C. asiatica* of India (RGR 0.82 g. g^{-1} $month^{-1}$; Singh and Singh 2002). That could be due to variation in environmental condition as well as in genotype. RGR for species grown under more or less optimal conditions may be as large as 10.5$g.g^{-1}$ $month^{-1}$ (Grime and Hunt 1975). RGR of *C. asiatica* was lower than this rate.

Low relative growth rate in plantlets of samples from open agricultural land could be the reason for open habitat. Due to direct solar radiation causing low moisture, high temperature and less nutrient content in soil, open agricultural land is in relatively stressful condition for this plant species. Species adapted to environments with growth limiting conditions like nutrient-poor soils tend to have an inherently low RGR (Grime and Hunt 1975). Nutrient poor habitat possibly led to species that have a relatively large investment in leaf biomass per unit of leaf area (low SLA). A comparatively low SLA may be advantageous in such habitats when this is caused by extra investment in

secondary metabolites, for instance phenolic compounds which may give protection under unfavorable circumstances (Waterman and McKey 1989).

6.6 Phenological Patterns

Phenophases are the result of assembly between a variety of selective pressures such as seasonal climatic changes, resource availability, and the presence of pollinators, predators and seed dispersers (Fenner 1998). In *Centella asiatica*, flowering began when soil was moistened by few showers of pre-monsoon rain and temperature increased, with flowering peak during early monsoon (Fig 5.9). This pattern is common in seasonally dry tropical region, where flowering is induced by rainfall and often concentrated in transition from the late dry to the early wet season (Rathche and Lacey 1985, Murali and Sukumar 1994). Early flowering in open agricultural land could possibly be due to direct light availability as well as less density of plants. Resource availability influences the timing of phenological events (Rathche and Lacey 1985). Plants grown in low light condition tended to spend more resources on growth of vegetative part like leaf area, petiole length and internode length rather than reproductive growth (Evans 1972). That could be the reason for relatively less number (i.e 42 %) of individuals in reproductive stage even during peak flowering season in shady habitat. Moisture, light and the presence of adequate reserved food could have imposed bottom-up selective pressure on the timing of flowering (Elzinga *et al.* 2007). The plants in shady habitat could favour vegetative reproduction than sexual ones.

Centella asiatica had short (about one month) flower-to-fruit duration after maturation, seeds were released out soon. Seeds of *C. asiatica* had no active mechanism of dispersal. It dispersed by surface runoff water during rainy season and through wind. Though most of herbs of moist tropical regions have fruiting peak during post-monsoon (Bhat and Murali 2001), *C. asiatica* had fruiting peak during mid monsoon. Since seeds of *C. asiatica* were very small in size (average length 2.8mm, breadth 1.8 mm, and mass 1.34 mg/seed), this could help seeds to float on surface runoff water in rainy season (Howe and Smallwood 1982).

Centella asiatica showed spatial variation in timing of phenological events. In open habitat this plant started flowering and produced fruits earlier than at other sites. This pattern of difference between different habitats was a common feature in tropical forest

at community as well as species level (Newstrom *et al.* 1994). Seeds began to germinate after 2-3 months after maturation.

6.7 Germination Ecology

Variation in seed characters in different population of *Centella asiatica* could possibly be due to different environmental conditions in the habitats where the plants were collected from. Seed mass of Pokhara, Dhampus, Lalitpur and Gorkha were significaly smaller than that of other sites. Low seed mass of these samples could be due to shady habitat of collection site (Table 3.1). Clonal plants propagated vegetatively by vegetative offshoots during suitable environmental condition. Plants allocate more resources in vegetative growth rather than in sexual reproduction i.e. for development of seeds (Evans 1972). So seeds that developed in shady habitats might not store much reserve food materials for developing embryo.

In the present study, the freshly collected seeds had the highest viability which declined progressively as the duration of storage increased. In general, deterioration of seeds with ageing results in the loss of viability and vigour which is usually due to the alteration in moisture content, changes in biochemical composition and increased leaching of electrolytes and low molecular weight substances during storage (Kalpana and Rao 1991, Hartmann *et al.*1997).

Pre-treatment significantly affected the mean cumulative germination of *Centella asiatica* (Table 5.5 & Fig 5.11). GA_3 treatment induced early germination by two weeks. This showed that the treatment was effective in inducing metabolic activity in the embryo required for the initiation of germination process (Groot and Karssen 1987). In contrast, dipping seeds in warm water (60ºC) reduced germination from 68% to 38%; this could be due to the destruction of certain enzymatic constituents present in the seed. Gill (1996) attributed the major cause of loss of viability at high temperature to the scarcity of oxygen since water has less solubility at high temperature. However Pre-treatment of seeds by 10% HNO_3 and warm water (30ºC) hastened the seed germination of *C. asiatica* by one week earlier than the control (Table 5.5); these treatments might have enhanced the metabolic activity required for germination. Jha and Jha (2006) also reported better germination of seeds of *Alysicarpus vaginalis,*

Desmodium triflorum and *Axonopus compressus* when treated with conc. HNO_3 for 10 minutes than in control.

Significantly higher percentage of seed germination of *C. asiatica* was recorded in light than in dark (Fig. 5.12). Seed germination was better in white and red light than in far-red, blue light and in dark, as reported for some other weed species (Kiatsoonthorn and Tjitrosemito 1992, Bell *et al.*1999). Photoblastic seeds germinate better in the open than under the forest canopy (Olatoye 1965). In the open, solar radiation comes unhindered; moreover associated with this is increase the air and soil temperature. This best explain the luxuriant growth of the seedlings of *C. asiatica* in the open land especially along the road sides and lawns at the onset of the rainy season (April-May) in Nepal. Thus, *C. asiatica* seeds may not germinate well under a plant canopy where the FR/R ratio is high as suggested by Frankland (1981). The mechanism of red/far-red light regulating seed germination via phytochrome system is well understood (Cone and Kendrick 1986). The inhibitory effects of blue light and dark condition on germination of weed species have also been reported by Jha and Jha (2006).

Taketay (1998) has reported germination in some photodormant *Solanum* species in the dark under the average amplitude of temperature fluctuation around 17ºC. In the present study seeds of *C. asiatica* germination was inhibited in the dark under the average amplitude of 10ºC. Least germination in the dark indicates that only some *C. asiatica* seeds can germinate even below the soil depth, or without light induction. This observation suggests that photo-dormance (no white light induction) is not present in *C. asiatica* seeds.

Germination of *C. asiatica* decreased with an increase in salinity, and was substantially inhibited at 6500 ppm NaCl (Fig 5.13). Maximum germination was obtained in the control. The result agrees with the work of Mondal *et al.* (1988) and Karim *et al.* (1992). Salt induced inhibition of seed germination could be attributed to osmotic stress or to specific ion toxicity (Huang and Redmann 1995). It has been assumed that in addition to toxic effects of certain ions, higher concentration of salt reduces the water potential in the medium which hinders water absorption by germinating seeds and thus reduces germination (Maas and Nieman 1978, Welbaum *et al.*1990). Germination decreased significantly as the level of salinity of the medium increased due to decrease in water movement into the seeds during imbibitions (Mauromicale and Licandro 2002,

Gulzar *et al.* 2001, Hadas 1977). This shows that highly saline soil i. e more than 6500 ppm is not good for cultivation of *C. asiatica*, as the seeds of plant could not germinate in such soil.

Aqueous extracts of *Parthenium hysterophorus*, *Chromolaena odorata*, *Ageratum conyzoides*, and *Xanthium strumarium* exhibited significant inhibitory effects on seed germination of *Centella asiatica* (Table 5.6). Similar findings were made by Oudhia (2000), who reported negative effect of *Lantana camera* leaf extract on germination of *Melilotus alba*. In present study the minimum inhibitory effect was recorded for *Chromolaena odorata* (1%), followed by *Ageratum conyzoides* (1%) and *Xanthium strumarium* (1%). Inhibitory effect increased with an increase of extract concentration indicating that the effect of plant extracts was dependent on their concentration. Similar observation was made by Ballester *et al.* (1982) in *Erica vegans*, *Callunga vu*lgaris and *Daboecia cantabrica*.

The inhibitory effects of studied plant species is caused by allelopathy. Many species of weeds produce toxins that are inhibitory to other species (Rice 1974). The inhibitory effect of these species on germination has been attributed to phytotoxic chemicals released from the leaf litter and roots. Kanchan and Jayachandra (1979) found the allelopathic potential of many weed species on various field crop species in India. Inhibitory effects of leaf leachates of *Parthenium hysterophorus, Chromolaena odorata, Ageratum conyzoids, Xanthium strumarium* on germination and growth of various weeds and crops have also been reported earlier (Jha *et al.*1996, Tefera 2002). This could occur only when some allelochemicals present in the leaf extract prevented growth of embryo, or caused the death. Though *Centella asiatica* plants grow along with alien plant species like *P. hysterophorus, C. odorata, X. strumarium* and *A. conyzoids* in the same habitat, the inhibitory effect of these species on germination of *C. asiatica* seed may have negative effect on its population in nature. This best explains the very low density of *C. asiatica* in *P. hysterophorus* invaded site than in non invaded site (Karki 2009).

6.8 Effect of Environmental Factors on Growth

6.8.1 Effect of Moisture Level

Drought stress is one of the important growth limiting factors for *Centella asiatica*. Significant effects of moisture level were observed upon most of vegetative traits of *C. asiatica*. A significant effect of different moisture level on leaf morphological characters such as petiole length and specific leaf area (SLA) was noted. The largest leaf area was recorded at 125% field water capacity (FWC). Leaf area decreased with increasing the water stress with the minimum leaf area observed in the 30% field water capacity. These results are consistent with the work of Verasan and Ronald (1978) in corn. Reduction of leaf area by severe water stress can be considered as an adaptive mechanism which helps to reduce water loss from the plant (Turk and Hall 1980). Longest root (10.23 cm) was observed in plants grown at 30% FWC. Under water stress the roots penetrated the deep soil, possibly providing greater access to the little water available and promoting production as a result. This might be the reason why *C. asiatica* grown at 30% FWC had the longest root.

Dry matter content of plants differed significantly (p = 0.001) among water stress treatment with the highest yield (1.04 g/ramet) at 100% . The total dry weights of plant decreased with exposure to high moisture stress (30% FWC) or excessive water (125% FWC). This could be the result of a reduction in chlorophyll content and, consequently, photosynthesis efficiency, as reported by Abdul-Hamid *et al*. (1990) in mungbean and by Castonguay and Markhart (1991) in common bean and terapy bean plants. The highest value of dry mass was obtained in plants grown at 100% FWC followed by 70% FWC. Significant reduction in fresh and dry matter content, and yield of another medicinal plant, *Mentha arvensis* due to water stress had been reported by Misra and Shrivastava (2000). The higher value of crop yield obtained at 100% FWC might be due to the presence of adequate moisture in active zone of root, with subsequent better utilization of nutrients. Under arid and stressed condition, overall plant growth was reduced as a result of both biochemical disruptions and reduced cell enlargement, which in turn led to reduced leaf expansion, total leaf area, and therefore reduced whole plant photosynthesis (Graan and Boyer 1990, Layer and Boyer 1992). That might be the reason for low value of growth traits and yield of *C. asiatica* grown at 30% FWC.

Lowest quantities of leaf N and chlorophyll were found in *C. asiatica* grown under low soil moisture (30% FWC, Table 5.7). Low moisture content in soil causes inability of plant to get all available nutrients in soil consequently causes low amount of water and nutrient in plant. The decrease of chlorophyll content in plant growing at FWC 30% may be associated with and most probably related to the decrease of plants water content (46.74%). It was stated earlier that the decrease of chlorophyll *a* content is highly related to the decrease of water content in plant leaves (Gadallah 1991, Wang and Nii 2000).

6.8.2 Effect of Soil Composition

All the measured traits of leaves varied significantly with soil texture however, the differences were only marginal for leaf area (Table 5.9). In present study, plants had relatively low SLA if grown on sand as well as on soils with excessively high clay content, which could be due to low amount of nutrient availability to the plants. Plants grown at low nutrient availability show a decrease in SLA (Sage and Pearcy 1987, Cunningham *et al.* 1999) partly as a result of the accumulation of non-structural carbohydrates or secondary compounds such as lignin or other phenolics (e.g. Waring *et al.* 1985). Greater SLA values indicate more leaf surface per unit biomass and, thus more area available for photosynthesis (Lambers and Poorter 1992), as found in this study for plants grown in 40% clay.

Growth of root was also affected significantly by soil texture. Under extreme water stress, the roots penetrated the loose-dry soil treatment more easily, as supported by the observed root depth. More macropores allowed root penetration, and soil particles were more easily pushed aside in the loose soil due to higher porosity, possibly providing greater access to the little water available and promoting production as a result. Low nutrient supply results in an increase in root length ratio (Boot and Den Dubbelden 1990, Ryser and Lambers 1995). Thus the longest root length in sand was attributed to low nutrient and water supply. Under stressed condition, overall plant growth was reduced as a result of both biochemical disruptions and reduced cell enlargement, which lead to reduced leaf expansion and total leaf area and therefore reduce whole plant photosynthesis (Graan and Boyer 1990, Layer and Boyer 1992). That is the reason for low value of growth trait data and low yield in pure sand. Further, inadequate contact of roots with the soil in sands could limit the uptake of water and

nutrients (Robbins 1946) which in turn, appears to reduce the growth rates in *C. asiatica*.

Dry mass of plant differed significantly among the soil texture (p = 0.035). It was higher in sandy loam soil (Table 5.10). Sandy loam soil with medium bulk density could have facilitated high biomass production. Compared to low and high bulk densities, the medium bulk density may provide longer retention of water in the soil and increase available water to plant due to the higher proportion of mesopores. Uptake of water and nutrients may also be improved by better root-soil contact (Bengough 2003). In contrast, the soil having high (100 %) clay and very low bulk density did not favor the growth of plants resulting low yield. Availability of soil resources, especially nutrients, critically influenced plant growth (Bazzaz 1996, Siemens *et al.* 2002). Hence, comparatively low biomass production in clay-rich soil suggests that *C. asiatica* had slower growth rates under soil having low available nutrient and air due to compactness of soil particles (i.e. high bulk density). Further, inadequate contact of roots with soil in coarse textured soil (i.e. in sand) could limit the uptake of water and nutrients (Passioura 1991), which in turn, appears to reduce the growth rate and yield of *C. asiatica*. On the other hand, comparatively high yield and growth vigour observed in sandy loam soil would enable the plant to pre-empt growth resources.

Soil textural type had significant influence (p <0.001) on the leaf N of *Centella asiatica* Lowest values of leaf N (1.26%) and chlorophyll (6.23 mg/g) content of *C. asiatica* grown in pure sand were due to poor nutrient (0.008 % N) and less moisture content in that soil. The decrease of chlorophyll content in plants growing in sand may be associated with and most probably related to the decrease of plants water content (43.75%). Decrease of chlorophyll *a* content is highly related to the decrease of water content in plant leaves (Gadallah 1991, Wang and Nii 2000). Higher leaf N (2.16%) and total chlorophyll content (25.43 mg /g) of *C. asiatica* grown in 40% clay was due to sufficient amount of air, nutrient and water.

6.8.3 Effect of Light Condition

Light intensity had significant influence on growth and morphological characters. The number of leaves of *Centella asiatica* grown under full sunlight was, on average twice the number in shade. Some other tropical species showed the similar response, as in *C.*

asiatica, which increased twice the number of leaves growing in gaps when compared to shade grown plants (Pooter and Hayashida-Oliver 2000). The shaded plants (50–70%) produced larger leaves, longer petiole and internode, in order to capture more light, probably because of a shade-avoidance mechanism (Ballaré 1999). It was shown that plants grown in lower light tended more resources on vegetative part growth like leaf area, petiole length and internode length rather than reproductive growth (Evans 1972) causing less number of flower buds in more shaded conditions.

The significantly lower specific leaf area (SLA) in high light grown *Centella asiatica* might be due to leaf anatomical differences brought about by low quantum flux density as suggested by Lambers and Poorter (1992) and reflects a strategy to increase competitive ability of this species under low light through an increase in leaf area. The increasingly higher values for SLA in more shaded conditions could be due to the increase in leaf area and a reduction in thickness caused by shading. Leaves in the sun are usually thicker than those growing in the shade (Nobel 1983). An increase in SLA is a common response observed in plants grown under low light conditions (George and Nair 1990, Buisson and Lee 1993, Stoneman and Dell 1993).

In the present study, plantlets grown under full sunlight, had higher dry weight of roots in comparision to the aerial part (Table 5.12). A reduction in SLA and higher translocation of photoassimilates to roots were observed. Young plants of *Garcinia mangostana* showed reduced leaf area and higher dry weight translocation to root system under decreasing shading conditions (Wiebel *et al.* 1994). As suggested elsewhere (Thompson *et al.* 1992), the lower allocation to roots under low light conditions is known to be maximized in sun-loving plants, and probably reflects a response to attributes that improve carbon gain under reduced irradiance such as an increase in SLA, or that reflects a light seeking strategy such as an increase in petiole length and internode length. A common response to shade reported in many studies is a reduced allocation to roots (Zollinger and Kells 1991, Thompson *et al.* 1992, Messier 1992).

Leaf chlorophyll was found to increase with increasing shade up to 70% (Table 5.11). Leaf chlorophyll levels are controlled by light (Kramer and Koslowski 1979). In elevated radiation intensities, chlorophyll molecules are susceptible to photo-oxidation and the equilibrium is reached in lower radiation levels (Alvarenga *et al.* 2003). This

was the reason for having higher chlorophyll levels in shaded leaves than leaves grown under full sunlight. Higher chlorophyll concentration in shaded seedlings of *Guarea guidonia* was also reported by Alvarenga *et al.* (1998). There was significant positive correlation ($r^2 = 0.325$, $p = 0.003$) between leaf N with SLA. This may be the reason why plants grown in more shaded conditions had higher N content. This response follows the pattern reported by other studies in tropical species (Thompson *et al*.1992).

6.8.4 Effect of Integrated Manuring

All measured morphological traits of *Centella asiatica* were significantly higher in integrated manuring system than in individual application and control. The morphological traits like petiole length, stolon length and flower number per ramet, were higher in the 50/50: Urea/FYM treatment than in the other treatments. The number of runners and flowers on strawberries in plots grown with food and paper waste vermicompost was reportedly higher ($p \leq 0.05$) than on those grown with inorganic fertilizers only (Arancon *et al.* 2004). Application of organic manures significantly increased levels of organic C and N and the formation of water-stable aggregates, as compared with application of chemical fertilizers (Adrien 2006). The increase of N uptake appeared to be more obvious when the FYM was mixed with the mineral N fertilizer as compared to the 100% FYM or 100% N mineral fertilizer. It can be either due to the effect of FYM and mineral N fertilizer on improving soil physical properties, or to a higher mineralization of FYM which was due to mineral N inputs. Integrated manuring increased availability of macro- and micro-nutrients to plants, which led to high vegetative growth, and more absorption of nitrogen (Roe and Cornforth 2000). Role of organics in increasing yield of *C. asiatica* could be attributed to supply of all essential nutrients due to continuous mineralization of organic manures. Phytohormones extracted from FYM help the plant to grow more luxuriously even with reduced doses of chemical fertilizers (Saraf and Tiwari 2004). This might be the reason for higher value of vegetative and reproductive traits of *C. asiatica* grown under integrated manuring system than in individual application.

Integrated manuring system had significant positive effect on dry mass of *Centella asiatica*. It was significantly higher in 50/50 : Urea/FYM followed by 75/25 : Urea /FYM and 25/75: Urea/FYM. The existence of favorable nutritional environment under the influence of FYM and inorganic fertilizers had a positive influence on vegetative

and reproductive growth, which ultimately led to realization of higher yield. It was reported that the soyabean yield in application of 40 Mg ha^{-1} SS (sewage sludge) enriched with half chemical fertilizer increased in comparison to other fertilizer treatments (Pirdashti *et al.* 2010). The potato yield was significantly higher in the compost treatment than in the control, and comparable to that produced with 200 kg N ha^{-1} mineral fertilizer (Fragstein and Schmidt 1999). Yield and yield components of *Abelmoschus esculentus* were enhanced by the application of a combination of cowdung and NPK although there was no significant response to any of the treatments (Okwuagwu *et al.* 2003).

It seemed that there is a need for adding organic manures to the soil in combination with inorganic fertilizers, which increased the availability of nutrients considerably resulting in positive effect on growth and yield of plant. These results are in agreement with the findings of Babalad (1999) in soybean, who have opined that there is a need of organic application along with inorganic fertilizers.

Significantly higher value of leaf N and chlorophyll content was observed in integrated manuring system (Table 5.13). That was due to more N available to the plant during the integrated manuring system (Das *et al.* 2007). Nitrogen supply has large effect on leaf growth because it increases the leaf area of plants and, on that way, it influences photosynthesis. Photosynthetic proteins represent a large proportion to total leaf N (Field and Mooney 1986, Evans 1989). Chlorophyll content is approximately proportional to leaf N content (Evans 1983). That was the reason for having higher value of leaf N and chlorophyll content in treatment 50/50: Urea/FYM followed by 75/25: Urea/FYM and 25/75: Urea/FYM. Similar type of result was also reported in other plants. In soyabean chlorophyll content of leaf in combined application of 40 Mg ha^{-1} MWS (municipal solid waste*)*, VC (vermicompost), and SS (sewage sludge) were higher as compared to chemical and other organic fertilizers (Pirdashti *et al*. 2010). It was reported that leaf chlorophyll content in 20/80 (vermicompost/feedstock) treatments were significantly higher than in pure vermicompost (Ali *et al.* 2007). Higher chlorophyll concentration in leaf of maize and sorghum in integrated manuring of inorganic fertilizer with poultry manure than in individual application of manure was reported (Amujoyegbe *et al.* 2007). Due to significant differences observed in vegetative growth of *Centella asiatica* among different nutrient sources, the integrated manuring seems to be the most suitable for agricultural purposes.

6.9 Nutrient Content

Nitrogen content in leaves is the most commonly used index of nutrient status in plant body (Chapin and Cleve 1989). Leaf N content of *Centella asiatica* (1.76%) lies within the range (0.2–6.4%) of global data set for 2548 species compiled by Wright *et al.* (2004). It was comparable to leaf N content of some tropical herbs in Nepalese grasslands (0.9–2.3%, Jha 2003). Leaf N content from 1.5 to 2.0% (15–20 mg/g) has been considered as adequate for the growth of most plants (Chapin and Cleve 1989). Leaf N content of *C. asiatica* was within this range. The difference between N content of leaves and soil was large (i.e. high leaf to soil N ratio) (Table 5.15). The larger difference was probably due to low N concentration in the soil. It reveals that *C. asiatica* can grow well under low N condition.

The soil of *Centella asiatica* habitats had pH 5.63, total N 0.182%, organic carbon (OC) 3.16% with C/N ratio 17.91 (Table 5.15). Acidic nature of soil of study site may be due to high annual rainfall in temperate to tropical region of Nepal (Devkota and Quadir 2006) as rainwater containing dissolved carbon dioxide from air or from soil is effective in dissolving and leaching calcium from the soil.

Soil OC of the present study sites lies within average values (1.34–3.35%) reported for soil of tropical zone of eastern Nepal (Jha 2003). Average soil N content of the study sites was lower than the global average of soil N content which is 2 g/kg (0.2%) (Larcher 1995), while the value was close to soil N content of warmer climates (tropical and subtropical) where soil N is generally >0.1 (e.g. Banarjee *et al.* 1989, Paudel and Sah 2003, Jha 2003). Low C/N ratio (10 or smaller) in soil organic matter generally indicates an advance stage of decomposition and resistance to further microbial decomposition. A wide C/N ratio (35:1 or more) indicates little decomposition, susceptibility to further and rapid decomposition and slow nitrification (Tamhane *et al.* 1964). The average C/N ratio of the soil of *C. asiatica* habitats is higher than upper limit of C/N ratio of for fertile soil with stable organic matter (10–12, Biswas and Mukherjee 1999).

6.10 Phytochemical Constituents

6.10.1 Wild *vs* Transplanted

Main function of plant secondary metabolites is thought to be the adaptation of plants to their environment (Kliebenstein 2004). Environmental factors such as light, temperature, CO_2 availability, soil conditions, etc. have a prominent effect on secondary metabolism resulting in extreme variability in the phytochemical contents of wild/cultivated plants and the products derived from them (Kirakosyan *et al*. 2003).

HPLC analysis revealed considerable variability in the contents of active components among samples (Tables 5.16). Asiaticoside in samples from central Nepal ranged from 0.24 to 8.13 %, with a mean value of 1.88 % (Table 5.18). The mean asiaticoside content of samples from central Nepal (1.88 % d.w) is comparable to *C. asiatica* from Madagascar (Randriamampionona *et al.* 2007), in which the amount of asiaticoside ranged from 2.67 to 6.42% (d.w.). Comparative study within 10 ecotypes from various regions in India showed a correlation between genomic diversity and asiaticoside contents (Das and Mallick 1991) and the highest amount of asiaticoside was 0.11% d.w. Gupta *et al*. (1999a) also reported variable asiaticoside contents in five lines of *C. asiatica* collected from a field trial in India, mean values varied from 0.42 to 1.17%. Low quantity of asiatic acid and chicoric acids were recorded in all analyzed samples. . Generally, all *C. asiatica* samples showed relatively higher amount of asiaticoside than asiatic acid. This is in accordance with large amount of triterpene glycosides and trace of triterpenic acids from plants of Thailand, Costa Rica and Bahamas (Booncong 1989). However, high asiatic acid content was reported in *C. asiatica* of Malaysia (Pick Kiong 2004). The observation in this study is in agreement with the statement by many researchers that *C. asiatica* collected from different locations produced different amount of triterpenes. Apart from the environment, climate and soil condition, the method of extraction could also be a contributing factor for the diverse compounds in *C. asiatica* from various locations (Booncong 1989).

In *Centella asiatica*, concentration of asiaticoside and quercetin -3-O-glucuronide differed significantly with habitats. Significantly higher amount of the phytochemicals was measured in samples collected from open agricultural land and least from shaded grassland (Table 5.17). The plant growing in open agricultural land is possibly under

stress due to direct sunlight and less availability of moisture content. Perhaps due to increased solar radiation and temperature, the plants produced more secondary compounds in relation to the adaptation mechanism. Odabas *et al.* (2009) hypothesized that the high photosynthetic activity under high light intensity resulted on increased amount of carbon assimilation and enhanced the concentration of carbon-rich secondary metabolites in leaf tissues. In this study, soil analysis of open agricultural land revealed relatively low nutrient content (C and N) in soil compared to shady habitat. It is possible that this low soil nutrient is responsible for reduced growth traits in the study species (Table 5.1). Nutrient stress generally reduces growth more than it reduces photosynthesis *per sec* (McKey 1979) and thus, it has been argued that the expected surplus of carbon can lead to an accumulation of carbon-based secondary substances under such circumstances (Bryant *et al.* 1983). This might be the reason for having low amount of secondary metabolites in shady grassland with relatively high soil nutrient contents.

The altitude of collection site of *Centella asiatica* and asiaticoside content were inversely related with higher contents of triterpene in samples collected at 150–600 m asl. Asiaticoside content decreased with increasing altitude. The maximum asiaticoside content was found at 600 masl. On the other hand, quercetin -3-O-glucuronide increased with increasing altitude and maximum at 1800m asl (Fig 5.19). Thus, a negative correlation was observed between altitude and asiaticoside contents, whereas the opposite variation occurred for quercetin -3-O-glucuronide (Fig 5.19). The amounts of flavonoids have previously been related to altitude by other authors (Ganzera *et al*. 2008, Rieger *et al.* 2008).

The amount of most of the measured secondary metabolites (asiaticoside, quercetin -3-O-glucuronide, chicoric acid and quercetin) seemed lower in transplanted samples than in wild (Tables 5.18 & 5.19). The inferior quality of cultivated samples than wild have been reported in other plant species; wild ginsing roots are 5–10 times more valuable than roots produced by artificial propagation (Robbins 1998). Plants in wild environment are in condition of stress and competition which perhaps would not be expressed under monoculture conditions (Schippmann *et al.* 2006). Active ingredient levels can be much lower in fast growing cultivated stocks whereas wild population can be older due to slow growth rates and can have higher levels of active ingredients

(Schippmann *et al.* 2006). Randriamampionona *et al.* (2007) reported 5–6 times lower amount of asiaticoside content in *in vitro* plants than in wild samples of *C. asiatica* of Madagascar.

6.10.2 Effect of Environmental Factors

Water stress had significant effect on asiaticoside and asiatic acid contents of *Centella asiatica.* Higher amount of asiaticoside and asiatic acid in 30 % FWC could be due to stress induced synthesis of more secondary metabolites. Earlier, Lim *et al*. (2006) reported that ginsenoides content of *Panax quinquefolium* increased due to water stress. Plants produce additional secondary metabolites as adaptive mechanism to protect it from stress condition (Edreva *et al*. 2008).

Soil texture had significant influence on concentration of measured secondary metabolites. It was significantly higher in sand (Table 5.20, B) than in other soil composition. The amount of asiaticoside was high (0.565 mg/g) in plants grown in sand; the amount was same as in wild samples. It could be due to low nutrient content (Table 4.1) in sand than in other soil types. Nutrient stress reduces growth, so as to protect plant from stress condition due to nutrient, accumulate more secondary metabolitres as adaptation to outer environment.

Light intensity had significant influence on asiatic acid content. It was significantly higher in 70 % shading. That might be due to stressful environment in terms of light availability. Plants produce more secondary metabolites in stress condition to protect from such environment. Briskin and Gawienowski (2001) reported a continuous increase on the level of hypericins in leaves of *Hypericum perforatum* with increasing light intensity from 106 to 402 mol $m^{-2}s^{-1}$. Each 70 to 100 mol m^{-2} s^{-1} increase in light intensity yielded about a 1.2 − 1.5 fold increase in leaf total hypericin level. Relatively higher amount of asiaticoside and quercetin -3-O-glucuronide were recorded in plants grown in full sunlight (Table 5.20, C). Least amount of these compounds was in samples from partial shading (30 and 50% shading). In partial shade conditions, plants might not be under stress, and invest resource to vegetative and reproductive traits, causing higher biomass production.

There was no significant difference in the phytochemical content in *Centella asiatica* treated with different N sources.

6.11 Essential Oil

The yield of essential oil of *Centella asiatica* from the three different habitats of Nepal ranged from 0.09 % (open agricultural land) to 0.12 % (shady grassland) with mean value 0.10 % (Table 5.21). Essential oil content was the highest in partially shaded environment where biomass production was also relatively high (Table 5.1). There is often positive correlation between biomass production and essential oil content in plants (Marchese and Figueira 2005). The present value of essential oil was higher than reported from South Africa for the same species (0.06%, Oyedeji and Afolayan 2005).

The quali-quantitative composition of the essential oils varied greatly among the samples grown in the three different habitats (Table 5.22) and only 18 compounds, such as γ-caryophyllene, caryophyllene oxide, β-caryophyllene, and spathulenol, were in common. In the essential oil of *Centella asiatica* from South Africa, the germacrane derivatives were among the predominat class of constituents (21.78%) (Oyedeji and Afolayan 2005), whereas in samples from Nepal, sesquiterpenoid hydrocarbons were the most abundant. In essential oil samples extracted in present study, 24 constituents out of 52 (Table 5.22) were identified in amount higher than 1%. Variation in essential oil constituents across the habitats could be due to variation in light condition. Accumulation of essential oil in herbs directly or indirectly depends upon light (Plummer *et al.* 1999). Although the light intensity perceived by the plants was not evaluated in present study, higher levels of canopy cover above plants generally result in higher levels of shading. In open conditions *C. asiatica* produced high amount of α-humulene and caryophyllene oxide, while in shaded area these compounds were produced in low amount. Studies on essential oil yield conditioned by shade levels have shown that each species responds differently to light intensity. In peppermint (*Mentha piperita*), highest oil yields, including the production of limonene, resulted from high photon flux density (Clark and Menary 1980). Quantitative differences in production of particular oil components are also seen in *Pinus monticola* where southern-facing branches have higher *α*-pinene, *β*- pinene, and limonene content than the more shaded northern-facing branches (Hanover 1966). The total oil concentration in *Pothomorphe umbellata* was the highest in the plants subjected to 30% shade (Mattana *et al.* 2010). Shading effects would also be compounded by differences in leaf temperature (Plummer *et al.* 1999), but this aspect was not explored in present study.

Studies on essential oil yield conditioned by shade levels have shown that each species responds differently to light intensity. In species such as *Thymus vulgaris* (Li *et al.* 1996) and *Matricaria chamomila* (Saleh 1973), essential oil content increased when grown under intense light. But in species like *Anethum graveolens* (Halva *et al.* 1992), *Salvia officinalis* (Li *et al.* 1996) and *Pothomorphe umbellata* (Mattana *et al.* 2010) gave higher essential oil yield when cultivated under shade. Variation in essential oil in different habitats could also be due to variation in soil nutrients and pH (Table 5.15, Alvarez-Castellanos and Pascual-Villalobos 2003).

Some of the compounds such as Thujopsene, α-terpineol, α- Pinene and camphene were detected in very small quantity (<1%) but other alcohol compounds such as linalool and cis-geraniol were detected in relatively high concentration (Table 5.22). Among these components α-terpineol is known for myorelaxant and antispasmodic effects (Magalh *et al.* 1998). Linalool is very important substance used in foodstuffs as a food additive (JECFA 1999, Anonymous 2000) and for various uses in pharmacology (Sugawara *et al*. 1998, Carson and Riley 1995). Geraniol has high relative ovicidal activity against human lice (Priestley *et al.* 2006). Limonene has also shown to have anti-cancer activity (Pamela *et al*. 1992). Camphor, an important constituent of essential oil of *C. asiatica* has shown antimicrobial potentials (Pattnaik *et al.*1997, Tzakou *et al.* 2001).

In conclusion, *C. asiatica* can be an important source of essential oils useful for the pharmaceutical, cosmetic and food industries. The oils were characterized by a high amount of sesquiterpenoid hydrocarbons mainly γ-caryophyllene, β-caryophyllene, β-farnesene and α-humulene.

6.12 Genetic Variation

Morphological traits of *Centella asiatica* measured in present study showed a significant variation among the 21 populations (Appendix 4). Length and width of leaf, and number of leaves and flowers increased with increasing plant biomass. Dendogram based on the morphological data revealed that most of the hill populations grouped into seperate cluster except Lamjung one, whereas terai and innerterai populations made distinct group (Fig. 5.19).

Most of populations of mid hills were grouped in cluster I and that of low lands were grouped in cluster II. This grouping was mainly based on leaf size, leaf margin, petiole length, branching, and the number of flowers per ramet. Thus two morpho-types (Plate 2) were clearly distinguished in *C. asiatica* populations of Nepal. Distinction of a similar morpho-type has also been reported among *C. asiatica* populations occurring in Sri Lanka (Anonymous 1978) and India (Mathur *et al.* 1999) where this plant has been cultivated commercially as a vegetable crop. Two cultivars, one with a small leaved creeping form, and the other with a large leaved erect bush form have been grown commercially.

In this study, 81% polymorphism was observed *C. asiatica* by using eight primers (Table 5.25). Padmalatha and Prasad (2008) reported 87% polymorphism among *C. asiatica* of India by using sixteen primers. The range of Jaccard's similarity coefficient ranges from 0.52 - 0.911 (Table 5.26). The highest similarity coefficient (0.911) was observed between populations of Kirtipur and Matatirtha (both lie in Kathmandu valley), which clearly depicts that genetically they are the closest among the 21 populations. That may be due to close geographical location (2 km apart) of these two populations. As *C. asiatica* is a clonal plant, it reproduces both through vegetative (ramets) and sexual (seedlings) means. Due to clonal nature it spreads widely and can cover wide range of area by a single parental clone. That might be the reason for highest similarity coefficient (i.e low genetic diversity) between samples of Kirtipur and Matatirtha area.

The dendogram based on RAPD data was also consitent with morphological ones and populations included in this study were distinctly gouped into hill and terai types (Fig 5.20). Despite the populations separated into two distinct groups, most of the populations shared higher degree of similarity. The higher similarity value might have resulted from clonal mode of propagation hindering the gene shuffling generation after generation through sexual cycle (Mar´ın *et al.* 2010). Analyses of phylogenetic tree based on RAPD marker ,Lamjung population seemed to be the more primitive and could be the founding member. The selection pressure and forces of evolution could trigger the divergence on this population and resulted arrays of genotypes with greater adaptation in diverse ecological niche.

Though dendograms from morphological attributes and RAPD shared a similarity in some respects, there is lack of perfect correlation. The lack of correlation between dendogram could be the result of point mutation/ insertion- deletion causing silent mutation in primers annealing sites in non-coding region of the *C. asiatica* genome. Hence the genes governing the phenotype produced same morphological attributes though the populations were diverged at the DNA level. The lack of correlation might also be resulted from clonal propagation and homogenous in growing environments. The absence of direct correlation between the morphological and genetic similarities was also observed for wild populations of other plants viz. *Lotus corniculatus* (Steiner and Santos 2001), *Trifolium repens* (Greene *et al*. 2004). However, to verify this hypothesis, use of more polymorphic and functional markers such as SSRs, STSs, ESTs and SNPs (Xu 2010) are suggested.

In conclusion, result of present study demonstrates that there are two morphotypes of *Centella asiatica* in Nepal. The similarity coefficient value 0.52 - 0.911 revealed that moderate genetic diversity among the *C. asiatica* population. It demonstrates that RAPD marker is an useful tool for investigating genetic diversity assessment of *C. asiatica* population. It is strongly recommended that both morphological and molecular assays be used as complementary methods in describing the population diversity in the populations. However, it is also worthwhile to study further with more exhaustive sampling of populations and advanced molecular markers technologies.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Centella asiatica grows in a wide range of habitats from open grass land, grassland under shaded to agricultural land from 85 to 3300 masl in Nepal. Density, growth traits and yield varied significantly with habitat types. In terms of density and plant biomass and area-based biomass yield, partially shaded grassland was the most suitable natural habitat of *C. asiatica.* Number of flowers per ramet, fruiting frequency and seed set were higher in open agricultural fallow land.

Seed viability declined with storage duration. Pretreatment like soaking seeds in GA_3 shortened the time required for germination initiation while the aqueous extract of some invasive plants viz. *Chromolaena odorata, Parthenium hysterophorus, Ageratum conyzoides* and *Xanthium strumarium* had inhibitory effects on germination which threaten the population density of *C. asiatica* in nature.

Regenerative potential of *C. asiatica* through sexual reproduction was relatively low in densely populated, shady site, because less than one half of mature individuals entered into reproductive phase and less than one tenth individuals developed fruits. Pot experiments showed that growth of *Centella asiatica* was significantly affected by light, soil composition, moisture content and nutrient condition of soil. It showed better growth and higher yield under partially shaded (30%), sandy loam soil, adequate moisture (100% FWC) and integrated manuring (organic and inorganic fertilizer) conditions. This indicates that the plant grows well in mesic environment.

Essential oil yield of *Centella asiatica* was higher in partially shaded habitat than in open habitat. *C. asiatica* can yield an essential oil useful for the pharmaceutical, cosmetics and food industries for its high content of compounds especially (E) *β*-farnesene, *β*-linalool, germacrene, *β*-caryophyllene and *β*-elemene.

Two distinct morphotypes of *Centella asiatica*, one with small leaves, dentate to serrate margin with creeping habit and the other with large leaves, crenate to entire margin with erect habit was clearly distinguished in Nepal. Similarity coefficient among population ranged from 0.52–0.91 indicating a moderate level of genetic diversity in *C. asiatica* of Nepal.

High concentration of selected secondary metabolites and better growth performance of *C. asiatica* was recorded in central Nepal than in eastern and western region. Elevation also affect concentration of secondary metabolites.Concentration of asiaticoside was higher in samples collected from lower altitude (600 m asl) while concentration of quercetin-3-O-glucuronide was higher at higher altitude. Significantly higher amount of secondary metabolites was recorded in open agricultural fallow land than in other habitats. Wild samples had higher secondary metabolites than in transplanted samples,

Based upon the present study, the two hypotheses of the present study have been accepted. Morphology, genetic character and active phytochemicals in *Centella asiatica* at different habitats and ecological regions of Nepal varied, and supported the hypothesis that *Centella asiatica* has intraspecific variation. Variation in phytochemical constituents at different ecological regions supported the hypothesis that phytochemical constituents in *Centella asiatica* is governed by environmental condition.

7.2 Recommendations

Following recommendations have been made based on empirical data generated during the present study.

1. Due to varied geographic nature of country, there is high chance of variation of important chemical constituents among the plant populations at different locations; so extensive research on quantification of important chemical constituents in Nepalese medicinal plants should be undertaken.
2. Due to high terpene contents in samples from Gorkha and Chitwan districts, germplasm of *C. asiatica* from these two locations has been recommended for conservation and used as starting materials for cultivation of this plant in Nepal.

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APPENDICES

Appendix 1. Duncan test for mean comparisons of different traits among different populations (transplanted) of *Centella asiatica.* [For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, α = 0.05).

Populations	No of primary branches	Pet iole Length(cm)	Leaf Length (cm)	Leaf width (cm)	SLA (cm^2/g)	Leaf Nitrogen (%)	Stolon Length (cm)	Leaf number	Flower umber	Seed number/Ramet	Mass of 100 seeds	Dry mass individual plant (g)	Total chl (mg/g)
CAJha	5.1^{e}±1.5	6.99^{j} ± 2.3	1.85^{c-e} ±0.21	3.27^{g-i} ± 0.23	525^{b} ±180	2.0^{a-c} ± 0.22	8.7^{d-f} ± 2.3	5.9^{bc} ± 1.15	14^{b} ±1.9	9.0±3.0	0.14±0.1	2.6^{gh} ± 0.84	16^{c} ±0.01
CAMak	4.12 ^{cd}±1.2	5.63^{d-g} ±1.3	1.95^{d-f} ±0.38	3.22^{g-i} ± .95	257^{a} ± 138	2.0^{a-c} ± 0.12	7.6^{b-d} ± 0.95	3.9^{bc} ± 0.96	14^{b} ± 2	10.0±2.0	0.09 ± 0.01	3.1^{i} ±1.3	18^{c} ±0.01
CADan	4.5^{d}±2.6	5.19^{g-i} ±1.46	2.3^{g} ± 0.37	3.22^{g-i} ± 1.0	204^{a} ±82	2.1^{c} ± 0.18	8.6^{d-f} ± 2.5	3.7^{bc} ± 1.29	14^{b} ± 4.7	12.0±3.0	0.13	2^{d-f} ± 0.9	14^{b} ±0.02
CABan	5.21^{e}±1.0	5.68^{i} ±0.83	2.02^{f} ± 0.38	3.25^{g-i} ± 0.5	230^{a} ± 100	3.1^{h} ± 0.1	10.1^{gh} ±1.8	3.5^{b} ± 0.76	19^{fg} ± 3	15.0±3.0	0.13	2.6^{gh} ±1.0	14^{b} ±0.01
CAKan	4.62^{d}±2.3	5.56^{hi} ± 1.5	2.02^{g-i} ± 0.35	3.24^{g-i} ± 0.6	253^{a} ±90	2.4^{ef} ± 0.7	8.9^{ef} ±1.8	5.1^{d} ± 1.5	15^{b} ±3.2	10.0±3.0	0.12	2.3^{gh}±0.5	10^{ab} ± 0.12
CADhan	2.54 ^{ab}±1.2	3.3^{b} ± 0.76	1.47^{b} ±0.1	2.57^{bc} ± 0.3	423^{a} ± 28	1.9^{ab} ± 0.13	5.6^{a} ± 0.85	2.5^{a} ± 0.64	19^{fg} ±1.8	15.0±3.0	0.09	1.6^{cd} ±0.4	12^{b} ± 0.12
CAKir	3.84^{c}±1.6	4.4^{d-g} ± 0.8	1.89^{c-f} ± 0.33	3.16^{f-i} ±0.3	489^{a} ± 32	1.84^{a} ± 0.28	7.6^{b-d} ± 0.7	3.6^{bc} ± 0.9	17^{c-g} ± 0.4	15.0±2.0	0.14	2^{e-g} ± 0.5	8.47^{a} ± 0.13
CAMata	2.41^{ab}±1.5	3.11^{b} ± 0.5	3.11^{b} ± 0.48	2.69^{cd} ± 0.19	577^{a} ± 33	2.3^{e} ± 0.8	8.2^{c-e} ±0.91	3.7^{bc} ± 0.63	18^{d-g} ± 2	15.0±6.0	0.12	1.4^{cd} ± 0.5	14.4^{b}± 0.21
CAGor	1.82^{a}±0.5	2.21^{a} ±0.5	2.21^{a} ± 0.48	2.36^{ab} ± 0.22	858^{b} ±95	2.53^{fg} ± 0.28	4.8^{b} ± 1.7	3.3^{d} ± 0.92	21^{i} ±1.6	18.0±3.0	0.09	0.7^{ab} ± 0.32	14.1^{b} ± 0.01
CaLam	5.41^{e}±2.1	15.15^{k} ± 2.	2.45^{g} ± 0.47	4.25^{j} ± 0.82	170^{a} ±77	2.24^{de} ± 0.2	10.5^{h} ±1.11	3.3^{c} ± 1.06	0.0 a	0	0	2.9^{h} ±0.4	7.5^{a}± 0.21
CAPok	2.21^{a}±0.5	3.86^{b} ± 1.0	3.86^{b} ± 1.0	3.05^{e-g} ± 0.5	225^{a} ± 47	1.98^{a-c} ±0.2	4.2^{a} ± 2.3	3.9^{bc} ± 0.6	21 ± 1.1	18.0±1.2	0.101	1.44^{cd} ± 0.3	14.4^{b} ±0.1
CaI	4.5^{d}±1.12	3.53^{b}± 1.0	3.53^{bc} ±1.0	2.78^{c-e} ± 0.21	244^{a} ±53	2.29^{e} ± 0.44	3.7^{a} ±1.5	2.3^{a} ± 0.5	15^{bc} ± 0	13.0±1.0	0.14	0.37^{a}±0.1	10.7^{ab}± .13
CApyu	3.21^{b}±0.5	4.08^{c} ±1.62	4.18^{c} ± 1.62	3.44^{i} ±0.48	496^{a} ± 66	2.47^{fg} ± 0.33	4.5^{a} ± 2.7	3.7^{bc} ± 0.9	21.5^{i} ± 0.5	15.0±3.0	0.12	1.39^{cd} ±0.3	13^{b} ± 0.1

Contd…. Appendix 1

CADam	2.01[a]±1.1	4.05[c-f] ± 0.99	4.25 [c-f] ± 0.99	2.9 [d-f] ±0.27	594[a] ± 46	1.96 [a-c] ±0.5	5.95 [bc] ± 1.3	3.7[bc] ± 1.12	17.9 [c-g] ± 0.4	12.0±3.0	0.098	1.36 [cd] ±0.68	19[c]± 0.21
CABar	3.42[b]±2.1	4.7 [e-h] ± 0.82	4.7 [e-h] ± 0.82	3.36 [hi] ± 0.52	218 [a] ± 86	2.3 ±0.14	8.87 [ef] ±1.6	5.2[d] ± 2.2	15.59 [bc] ± 0.6	11.0±2.0	0.143	2.18 [gh] ± 0.6	18[c] ± 0.21
CADham	3.01[b]±1.0	3.47 [bc] ± 0.7	3.47 [bc] ± 0.66	3.39 [i] ± 0.48	405 [a] ± 44	2.6 [e] ±0.11	8.9 [ef] ±1.4	3.1[bc] ± 2.0	12.25 [b] ± 2.5	9.0±2.0	0.011	1.69 [d-f] ± 0.2	13 [b] ±0.1
CASur	3.52[b]±1.2	4.36 [d-g] ±0.94	4.36 [d-f] ±0.94	3.07 [f-h] ±0.3	549[a] ±73	2.5 [g] ±0.3	6.1[b] ±2.9	3.9 [bc] ±0.9	24.6 [gh] ± 4	18.0±3.0	0.12	1.63 [c-e] ±0.31	14 [b] ± 0.12
CAChit	3.8[c]±2.1	5.09 [f-i] ± 1.0	5.09 [f-i] ± 1.0	3.38 [i] ±0.35	167 [a] ±55	1.9 [f] ±0.13	7.1 [b] ± 1.7	4.2[c] ±1.2	21 [de] ± 1.0	15.0±1.2	0.13	1.27 [c] ± 0.4	14.18 [b]± .1
CALalit	2.13[a]±0.8	3.3[b] ± 0.76	3.3 [b] ±0.76	2.57 [bc] ± 0.27	424 [a] ± 277	1.89 [ab] ±0.14	4.6 [a] ± 0.85	2.6[a] ± 0.7	12.4 [e-g] ± 1.5	8.9±2.3	0.098	1.55 [cd] ±0.39	13[b]±0.12
CaKai	4.2[d]±1.3	6.99 [j] ±2.32	6.99 [j] ± 2.32	3.27 [g-i] ±0.23	224 [b] ±180	1.99 [a-c] ± 0.22	8.7 [d-f] ± 0.23	3.9[bc] ± 1.15	12.62 [bc] ± 1.9	9.52±1.3	0.13	1.63 [c-e] ±0.84	17[c] ± 0.15
CASun	3.2[b]±0.8	4.56 [d-g] ± 1.8	4.56 [d-g] ±1.8	2.25 [a] ±0.2	283 [a] ± 4	2.04 [bc] ± 0.3	4.64 [a] ±1.6	4.4 [c] ±0.5	15.68[b] ±3	14.34±1.91	0.11	1.79 [b] ±0.44	18[d]± 0.15

Appendix 2. Collection sites and habitats of *Centella asiatica* specimens deposited at National Herbarium (KATH), Godawari, Lalitpur . All specimens were collected within Nepal. Some information from personal collection has been also presented.

SN	District	Locality	Elevation (m asl)	Eco -region*	Habitat	Herbaria
1.	Ilam	Phidim	1250	E	Open Grassland	**
2.	Jhapa	Bhadrapur	85	E	Fallow agricultural land	**
3	Sunsari	Inaruwa	96	E	Grassland	**
4.	Dhankuta	Hille	1800	E	Grassland	**
5.	Makwanpur	Daman	2300	C	Open Grassland	**
6.	Makwanpur	Hetauda	650	C	Grassland	**
7.	Chitwan	Gauriganj	250	C	Open Grassland, Agricultural Land	**
8.	Gorkha	Palungtar	600	C	Open Grassland, Agricultural Fallowland, Shady place	**
9.	Lamjung	Way to Besisahar	740	C	Shady grassland	**
10.	Lalitpur	Godavari	1550	C	Open Grassland	**
11	Kathmandu	Kirtipur	1350	C	Open and shady Grassland, Agricultural fallow land	**
12	Kathmandu	Matatirtha	1400	C	Shady Grassland, Open Agricultural fallow land	**
13	Kaski	Pokhara	850	C	Open grass land	**
14	Kaski	Dhampus	1800	C	Shady grassland, Agricultural fallow land	**
15	Pyuthan	Ampchaur	1250	W	Open Grassland, Shady grassland	**
16	Dang	Lamahi	150	W	Open grassland	**
17	Surkhet	Birendranagar	650	W	Shady grassland	**
18	Banke	Kohalpur	155	W	Open fallow land	**

Contd… Appendix 2						
19	Bardiya	Magaragadi	150	W	Shady grassland, Agricultural fallowland	**
20	Kailali	Dhangadi	130	W	Open grassland	**
21	Kanchanpur	Mahendranagar	150	W	Open grassland	**
22	Gulmi	Anghana	600		Shady grassland	KATH
23	Surkhet	Harraya Dada	3300	W	Open moist places	KATH
24	Surkhet	Solighopte	700	W	Open moist places	KATH
25	Salyan	Kalyan	870	W	Shady grassland	KATH
26	Lalitpur	Lele	1540	C	Open grassland	KATH
27	Sindhuli	Ratapur	1270		Shady grassland	KATH
28	Ilam	Ithung	2400	E	Open moist places	KATH
29	Baglung	Burtibas	1100	C	Shady grassland	KATH
30	Myagdi	Bhakiumle	1700	C	Open grassland	KATH
31	Morang	Tarahara	200	E	Moist soil around ditches	KATH
32	Pyuthan	Khalanga	1450	W	Open moist places	KATH
33	Bardiya	Katarnia	120	W	Shady grassland	KATH
34	Bardiya	Souha	600	W	On riverside of Babai	KATH
35	Sunsari	Sissoban	110	E	Forest gap of sissoo forest	KATH
36	Rolpa	Masina-kuchhap	850	W	Shady grassland	KATH
37	Rolpa	Liwang	950	W	Open moist places	KATH
38	Salyan	Koilalekh	1600	W	Shady grassland	KATH
39	Salyan	Ramdanda	1400	W	Open moist places	KATH
40	Salyan	Thar	2700	W	Shady grassland	KATH
41	Jumla		2300	W	Shady grassland	KATH
42	Dailekh	Chip-chipe danda	1090	W	Open moist grassland	KATH
43	Dang	Parsaini	650	W	Moist soil around ditches	KATH
44	Dang	Masine	2200	W	Open moist places	KATH
45	Jajarkot	Dasnera	1740	W	Shady grassland	KATH

Contd… Appendix 2						
46	Myagdi	Hagalegauda	1100	C	Open moist places	KATH
47	Parbat	Peepaltari	1050	C	Open moist places	KATH
48	Parbat	Kushma		C	Shady grassland	KATH
49	Parbat	Sika	2020	C	Open moist places	KATH
50	Baglung	Hatiya	1150	C	Shady grassland	KATH
51	Makwanpur	Tistung	2000	C	Shady grassland	KATH
52	Makwanpur	Hatiya	500	C	Shady grassland	KATH
53	Karepalanchok	Dapcha	1740	C	Shady grassland	KATH
54	Sindhupalchok	Sirubari	2500	C	Open moist places	KATH
55	Sindhupalchok	Balefi	1100	C	Open moist places	KATH
56	Dhading	Tharlang	1550	C	Shady grassland	KATH
57	Kaski	Deurali	2080	C	Open moist places	KATH
58	Kaski	Chitre	900	C	Shady grassland	KATH
59	Kaski	Pardi	920	C	Open moist land	KATH
60	Kaski	Bindabasini	900	C	Moist shady places	KATH
61	Kaski	Sikhi	2000	C	Moist places	KATH
62	Sindhuli	Kalapani	320	E	Open moist land	KATH
63	Dhanusha		110	E	Open moist land	KATH
64	Dolakha	Simigaun	1860	C	Partially shaded grassland	KATH
65	Gorkha	Taku	760	C	Partially shaded land	KATH
66	Gorkha	Thalajung	1400	C	Open moist land	KATH
67	Okhaldhunga		1730	E	On dry land	KATH
68	Dadeldhura		1250	W	Shady grassland	KATH
69	Kaski	Panchashe	1700	C	Open moist land	KATH
70	Taplejung		2296	E	Open Moist grassland	KATH

*W: Western Nepal, C: Central Nepal, E: Eastern Nepal. ** Collection of Anjana Devkota.

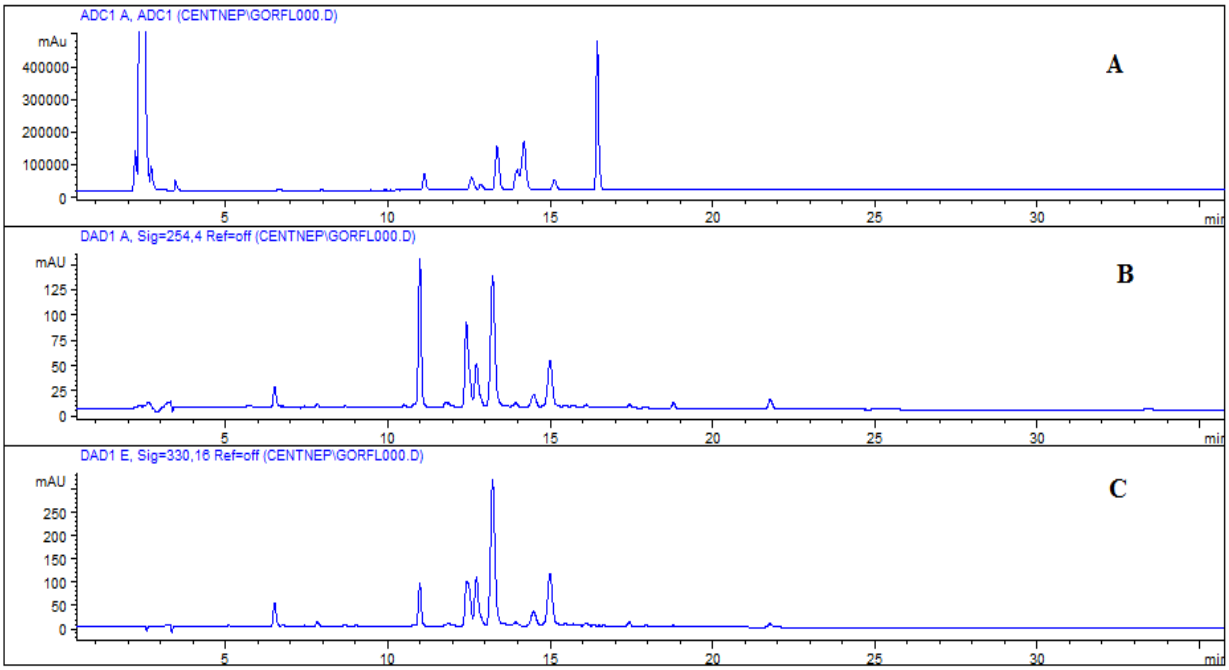
Appendix 3. Spearman's Correlation coefficient of soil attributes, plant biomass, and secondary metabolites of *Centella asiatica.*

	Density	Dry wt plant	Soil OC	Soil nitrogen	Soil pH	Asiatico-side	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Querce3-*O* glucuronide	Kaemp-ferol	Quercetin
Density	1												
Plant biomass	0.102*	1											
Soil organic carbon	0.13*	0.13*	1										
Soil nitrogen	0.16*	0.197*	0.936**	1									
Soil pH	-0.19	-.058	.022	.028	1								
Asiaticoside	0.29*	-0.103*	-.015	.030	-.290*	1							
Asiatic acid	-0.33	-.095*	-.155	-.170	-.171	.171	1						
Chicoric acid	-0.13	-.139	-.038	-.047	.108	-.047	.030	1					
Chlorogenic acid	-0.01	-.089	-.187	-.195	.038	-.230	-.520**	-.069	1				
Rosmaricnic Acid	-0.06	-.227	-.263*	-.277*	-.354**	-.167	-.269	-.066	.395**	1			
Quercetin 3O glucuronide	0.10	-.122	-.290*	-.290*	-.217	-.115	-.478**	-.053	.520**	.677**	1		
Kaempferol	0.01	-.123	-.188	-.188	-.129	.051	-.028	-.558**	.030	-.019	.162	1	
Quercetin	-.049	.183	.127	.140	.164	.097	-.025	-.067	.069	-.072	-.021	-.005	1

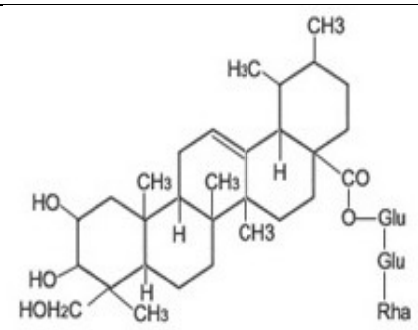
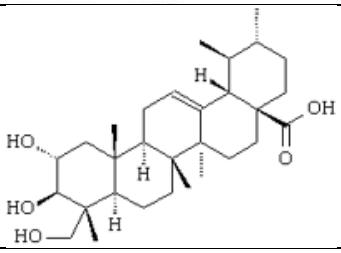
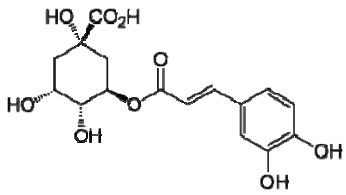
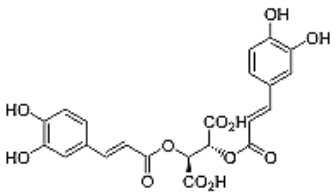
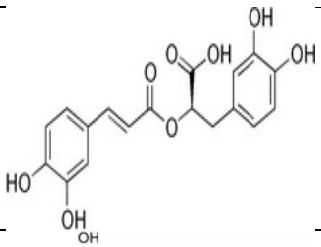
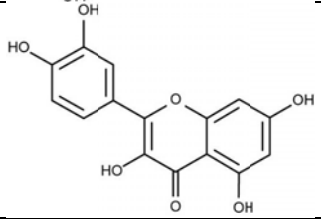
Appendix 4. Comparison of morphological characteristics between the twenty one accessions of *Centella asiatica* collected in various locations in Nepal and *C. asiatica* available at other localities (for CA01-CA21, N= 1890).

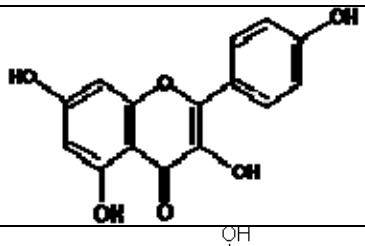
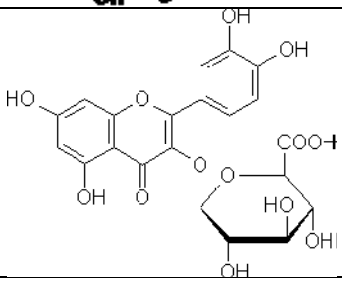
Location	Leaf length (cm)	Leaf width (cm)	Leaf Area (cm^2)	Petiole length (cm)	Leaf margin	Leaf shape	Reference
Nepal (CA01-CA 21)	1.22-2.66	1.9- 4.41	3.02-11.51	2.36-9.40	Crenate-dentate	Kidney, Heart	Collection samples of present study
Malaysia	2.0-4.0	3.0-7.5	5.89-29.46	5.0-19.0	Crenate with dentate based, lobed and crispate, dentate, cranulate, crenate	Kidney, heart and salad shape	Pick Kiong (2004).
Thailand/ North Carolina	2.5-7.0	2.5-7.5	6.13-51.55	2.0-13.0	Dentate	Kidney shape	Booncong (1989)
Costa Rica	2.5-7.0	2.5-7.0	6.13-48.11	2.0-13.0	Less coarsely dentate	Between kidney and heart shape	Booncong (1989)
Bahamas / Lousiania	5.5-7.0	2.5-3.0	13.50-20.62	5.0-25.0	Coarsely dentate at the base and less dentate on the upper margin	Heart shape	Booncong (1989)
C. asiatica (description by Solet *et al.* 1998)	1.0-3.0	2.0-4.0	1.96-11.78	5.0-19.0	Crenate	Kidney shape	Solet *et al.* (1998)

Appendix 5. HPLC chromatogram of selected samples of *Centella asiatica* recorded by ELSD (A), DAD at 254 nm (B) and 330 nm (C) respectively.



Appendix 6. List of analysed chemical components in present study and their molecular structure and activity.

Compounds	Molecular structure	Emperical formula	Molecular Weight	Bioactivity
Asiaticoside		C48 H78 O19	959.12152	Antioxidant
Asiatic acid		$C_{30}H_{48}O_5$	488.71	Neuroprotectant, inducer of apoptosis
Chlorogenic acid		$C_{16}H_{18}$ O9	354.095082	Antioxidant, inhibitor of tumor promoting activity, prevention of Type- 2 Diabetes
Chicoric acid		$C_{22}H_{18}O_{12}$	474.37	Antioxidant
Rosmarinic acid		$C_{18}H_{16}O_8$	360.31 g/mol	Antioxidant, Antibacterial, Antiviral, Antiinflammatory
Quercetin		$C_{15}H_{10}O_7$	302.236 g/mol	Anti-inflammtory, antioxidant

Kaempferol		$C_{15}H_{10}O_6$	286.23 g/mol	Antidepressant
Quercetin -3-*O* -glucuronide		$C_{21}H_{18}O_{13}$	478,37	Inhibition of lung cancer cell growth

Appendix 7 : List of Publications.

Research

1. **Devkota A.** and P.K. Jha.2008.Growth performance and Nutrient status of *Centella asiatica* (L.)Urban in different land uses of Kathmandu Valley, Nepal**.** ***International Journal of Ecology and Environmental Sciences***.34 (3):269-275.
2. **Devkota A**, S.Dall'Acqua, G. Innocenti, and P. K. Jha.2009. Quantitative analysis of secondary metabolites in *Centella asiatica* collected in different habitats**.** ***Chemia Analytica*.54:69-77.**
3. **Devkota A** and P.K Jha. 2009 a**.** Ethnobotanical study of *Centella asiatica* of Gorkha district. ***Nepal Journal of Forestry***. **13** (1).58-62.
4. **Devkota A** and PK Jha.2009 b**..**Variation in growth of *Centella asiatica* (L.) Urban along different soil composition. ***Botany Research International.2*** (1):55-60.
5. **Devkota A**, Stefano D A , Stefano C, Innocenti G and PK Jha.2010.*Centella asiatica* (L.) Urban from Nepal: Quali-quantitative analysis of samples from several sites, and selection of high terpene containing populations for cultivation. ***Biochemical Systematics and Ecology* 38:**12–22.
6. **Devkota A** and PK Jha.2010a. Seed Germination responses of *Centella asiatica.* ***Brazilian Journal of Plant Physiology* 22(2) :143-150.**
7. **Devkota A** and PK Jha .2010 b. Effects of different light levels on the growth traits and yield of *Centella asiatica.* ***Middle -East Journal of Scientific Research* 5(4):226-230.**
8. **Devkota A, S Dall' Acqua, P K Jha and G Innocenti . 2010.** Variation in the active constituent contents in *Centella asiatica* grown in different habitats in Nepal. ***Botanica Orientalis* 7:143-150.**
9. **Devkota A** and PK Jha.2011. Influence of water stress on growth and yield of *Centella asiatica* (L) Urban. ***International Agrophysics***. **25**(3) (***In press*).**
10. **Devkota A** and PK Jha. Effect of integrated manuring on Growth and yield of *Centella asiatica.* ***Tropical Ecology.(Under review*).**

11. **Devkota A , M Luchhin, HP Bimb and PK Jha .**Genetic diversity in different populations of Nepalese *Centella asiatica* (L.) Urban based on morphological and RAPD markers. **(*Draft*).**
12. **Devkota A**, S Dall' Acqua, S Comai , G Innocenti and P K Jha. Chemical composition of essential oil of *Centella asiatica* (L.) Urban from different habitats of Nepal. **(Draft).**

Review

1. **Devkota A.** and P.K. Jha.2008. Biology and medicinal characteristics of *Centella asiatica. Medicinal Plants in Nepal: An Anthology of Contemporary research,* (eds) PK Jha, SB Karmacharya, MK Chettri, CB Thapa and BB Shrestha. Ecological Society (ECOS). pp. 68-80.
2. **Devkota A.** and P.K. Jha.2010. Uses and Sustainable harvesting of *Centella asiatica* (L.) Urban. *Sustainable Use of Biological Resources in Nepal,* (eds) PK Jha, SB Karmacharya, MK Balla, MK Chettri and BB Shrestha Publisher : Ecological Society (ECOS), P.O. Box 6132, Kathmandu, Nepal. pp. 250-254.

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Growth Performance and Nutrient Status of *Centella asiatica* (L.) Urban in Different Land Uses of Kathmandu Valley, Nepal

ANJANA DEVKOTA* AND PRAMOD KUMAR JHA
Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal
* Corresponding author; *Email: devkotaa@gmail.com*

ABSTRACT

Centella asiatica (L) Urban is an important medicinal plant of tropical and subtropical belts of Nepal. Ramet density, stolon length, petiole length, specific leaf area (SLA), leaf nitrogen content, number of flowers in inflorescence and soil nutrients (nitrogen (N), organic carbon (OC) and organic matter (OM) contents of populations of *Centella asiatica* growing under different land uses (grazing, non grazing and agricultural land) in Kathmandu valley were recorded. Ramet density was highest in non grazing land with soil with 0.134 % N, 1.52% OC and 2.58 % OM. Leaves had 401.45 cm^2 g^{-1} SLA, 3.58 cm long petiole and 0.67 % N. The plants from the three sites differed significantly ($p<0.001$) in petiole length, SLA, leaf N, soil N, soil OC and soil OM contents. Thus land uses had significant effect on ramet density and leaf characters of *Centella asiatica* .Phenotypic plasticity in leaf petiole length and number of flowers per inflorescence appeared to be governed by light availability and height of associated species.

Key Words: Ramet Density, Petiole Length, Specific Leaf Area, Soil Characters, Phenotypic Plasticity.

INTRODUCTION

Medicinal and aromatic plants (MAPs) in Nepal are the important non-timber forest products (NTFPs) contributing significantly to the national economy. In Nepal about 70-80 %of populations in the mountain region depend on traditional medicines for health care (Manandhar 1996). About 1700 species of MAPs have been recorded from Nepal (Baral and Kurmi 2006).

Protection of wild population and cultivation for commercial purpose are two important strategies, which can prevent the species from becoming extinct. Cultivation is the best option to decrease harvest pressure on the wild population. However, there are some major problems in cultivation such as the decline in medicinal value of plants from cultivation (Hamilton 2003, Schippmann et al. 2002). A successful cultivation without any decay in medicinal value of the plant may need replication of wild habitat condition in the farm land, which is virtually impossible but can be maximized if we have detailed information on habitat requirements and plant growth performance in different habitats in wild condition. Biological study is a pre-requisite to develop a management plan for medicinal plants (Schippmann et al. 2002). However, detailed ecological studies on medicinal plants of Nepal are lacking except a few studies (e.g., Ghimire et al. 1999, 2005, 2008, Shrestha et al. 2007). This study was undertaken to understand the growth performance (ramet density and morphology) and nutrient status (leaf and soil) of *Centella asiatica* in three land uses in Kathmandu valley, Nepal. The specific objectives were to understand variation in density, morphology (vegetative) and nutrient status of plants and soil in different land uses.

Centella asiatica (L.) Urban (syn. *Hydrocotyle asiatica*; family Umbelliferae; Ghod tapre in Nepali) is an important medicinal herb of tropical to subtropical regions, growing in moist places up to an altitude of 2200 m, and also on moist stone wall or other rocky sunny areas. It is a small creeping herb with heart or kidney-shaped leaves emerging alternately in clusters at the nodes. Taxonomic description of this plant can be found in Flora of Bhutan (Grierson and Long 1999).

Traditional uses of *Centella asiatica* in different parts of Nepal have been well documented (Mahato and Chaudhary 2005, Shrestha and Dhillion 2003, Manandhar 1996, 2002). Most commonly the aerial parts of the plant are used against high blood pressure, gastritis, uric acid, fever and headache. People collect plants for their use from the wild but the plant is not cultivated in Nepal.

STUDY SITES

The Kathmandu valley (85º30' E to 85º 40' E and 27º55' N to 27º35' N, 1350 m above sea level) is roughly elliptical in outline and covers about 339 km^2 area. The area is drained by Bagmati River and its tributaries. The climate is warm temperate, but influenced by the tropical monsoon with wet summer and dry winter. Maximum and minimum temperatures range from 30ºC to 33ºC in summer and -3 ºC to 0 ºC in winter (DHM 2006). The valley is surrounded on all sides by hills and mountains (maximum elevation 2720 m, Shivapuri hill).The soil texture in the valley varies from loamy to clayey.

After reconnaissance survey three sites: Godavari (1600 m altitude), Kirtipur (1350 m) and Matatirtha (1600 m) were selected for the study. At each site three different habitats (land uses) i.e. grazing, non grazing and agricultural land (in fallow period) were selected for sampling population, plant material and soil. The sites were:

1. Non-grazing land was comparatively undisturbed and densely vegetated area where grazing was prohibited, but other human activities like cutting and clearing of vegetation occurred. This site was shaded by trees and shrubs like *Pinus roxburghii, Prunus*, *Rubus*, *Populus,* etc. Associated species were, *Chromalaena adenophora , Cynodon dactylon, Setaria* sp., *Geranium* sp.

2. Grazing land - where grazing pressure by cows and buffaloes was high all round the year. This site was open and received full sunlight. The associated species of *C. asiatica* were *Cynodon dactylon*, *Parthenium hysterophorus, Breea arvensis, Paspalum* sp.

3. Agricultural land in fallow period was open area receiving full sunlight and the vegetation cover was sparse. Grazing by cows was frequent but the grazing pressure was lower than in grazing land. Associated species were *Bidens pilosa*, *Cynodon dactylon*, *Hydrocotyle* sp., *Setaria* sp., etc.

MATERIALS AND METHODS

Field Sampling

Because of the plant having rosette habit, each adult with a rosette of leaves and a root system was considered an individual. Population sampling and leaf and soil sample collections were done during May 2007. Quadrats (1m×1m) were laid randomly in three different habitats (i.e. grazing, non- grazing and agricultural land). In each site thirty quadrats were randomly sampled in a large 40m×40m plot for ramet density. From each site ninety matured leaves were collected for measuring petiole length, specific leaf area (SLA) and dry weight of leaf. Altogether 270 leaves were collected for this purpose. Soil samples (200 g) were collected from each plot at rooting depth (5-10 cm) .Number of flower was counted in thirty inflorescences at each site; altogether ninety inflorescences were counted for this purpose. Leaf and soil samples both were air dried in shade for one week and stored in plastic bags until nutrient analysis. Stolon length of thirty plants at each site was measured; altogether, ninety plants were measured for this purpose.

Laboratory Analysis

Specific Leaf Area and Petiole Length

Petiole length, length and breath of leaves were measured .Then the leaves were oven dried (60ºC, 48 h) and mass of each leaf was weighed in electric balance (0.001g). Leaf area was determined by multiplying the product of length and breadth of leaves with a conversion factor (Zobel et al. 1987). The specific leaf area was calculated as the ratio of leaf area and leaf dry mass.

Leaf Nitrogen Content

Leaf nitrogen (N) content was determined by modified micro-Kjeldahl method following the procedure described by Horneck and Miller (1998). Thirty samples from each site were analysed.

Soil Analysis

Thirty soil samples from each site were analysed for soil organic carbon (OC), organic matter (OM) and nitrogen (N) contents. Air dried samples were passed through fine sieve (mesh size 0.5 mm) before analysis. Analyses were made following the methods described by Gupta (2000).

Numerical Analysis

The data were analysed for the significance of differences in measured attributes between the three sites by ANOVA and the Duncan's homogeneity test using SPSS (version 11.5, 2002).

RESULTS

Density

Three sites with different land uses differed significantly in density of ramets ($p<0.001$) of *Centella asiatica* (Table.1). Non grazing land had highest (76 plants m^{-2}) ramet density. Despite lowest ramet density, agricultural land had the highest number of flowers per inflorescence. At agricultural land, more than 90% of the ramets bore flowers.

Morphological variation

The average number of flowers per inflorescence was 9.83 (Table 1). The plants at three sites were different ($p<0.001$) in number of flowers per inflorescence. The number of flowers per inflorescence in agricultural land was four times and two times higher than in non grazing land and grazing land respectively. Specific leaf area ranged from 254.45 $cm^2 g^{-1}$ at agricultural land to 401.45 $cm^2 g^{-1}$ at non-grazing land (average 327.95 $cm^2 g^{-1}$).The difference in specific leaf area (SLA) among the sites was significant ($p=0.026$). Petiole length ranged from 2.4 cm at agricultural land to 3.58 cm at non-grazing land. The three sites differed significantly ($p< 0.001$) in petiole length. Average dry mass of a leaf was 19 mg and there was no significant difference ($p= 0.389$) among the sites (Table 1).

Nutrient content

Leaf N content ranged from 0.67 to 0.86% (average 0.75%). Three sites differ significantly ($p<0.001$) in leaf N content. Soil N content ranged from 0.134 to 0.24 %(average 0.182 %) and there was significant difference ($p<0.001$) among the three sites (Table 2). Soil organic carbon (OC) ranged from 1.16% at grazing land to 2.34% at agricultural land with average1.68% for all sites. Mean soil organic matter (OM) was 2.96%. There was significant difference ($p<0.001$) in soil OM and OC among the sites. C/N ratio ranged from 9.43 at agricultural land to 15.0 at non grazing land. There was no significant difference in C/N ratio among three sites.

DISCUSSION

Density

Land use pattern had significant influence on ramet density of C*entella asiatica.* The ramet density depends on stolon length; shorter the stolon length, higher is the ramet density. Generally, stolon is long and ramet density is high in nutrient rich sites (Tworkosk et al. 2001). But in present study stolon length was highest (5.91 cm) and ramet density was lowest (32 plants m^{-2})

Table 1. Density and morphological characters of *Centella asiatica* in Kathmandu valley. For each parameter significant difference between mean among different sites are indicated by different letters (Duncan homogeneity test, $\alpha = 0.05$). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[a]	Nongrazing Land	Grazigland	Agricultural Land	Mean	F value	P value[b]
Ramet density (no m^{-2})*	76 ±35.28b	66 ±28.7b	32 ±8.84a	58±0.03	23.21	<0.001
No of flowers/inflorescence*	4.65±7a	8.2 ±2.6b	17.07±6.56c	9.83±6.64	70.94	<0.001
Stolon length* (cm)	4.52±0.93a	4.63±1.27a	5.91±1.22b	5±1.6	12.77	<0.001
Petiole length$ (cm)	3.58±1.75c	3.06±1.32b	2.40±0.65a	3.01±1.4	17.84	<0.001
SLA$ ($cm^2 g^{-1}$)	401.5±486.6b	380.1±446.2b	254.5±151.3a	345.3±395.0	3.71	0.026
Dry mass per leaf (mg) $	20±0.015a	20±0.039a	16±0.009a	19±0.025	0.95	0.389

[a] Samples size (n): * n = 30, $ n = 90; b Bold number indicates significant difference among the mean, ± Standard deviation

Table 2. Leaf nitrogen (N) content and soil nutrients (nitrogen and organic carbon) *Centella asiatica* in Kathmandu valley. For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, $\alpha = 0.05$). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[a]	Nongrazing Land	Grazingland	Agricultural Land	Mean	F value	P value[b]
Leaf N (%)	0.67±0.05a	0.72±0.15a	0.86±0.09b	0.75 ± 0.13	25.94	<0.001
Soil N content (%)	0.134±0.23b	0.13±0.05a	0.24±0.12b	0.18 ± 0.08	10.867	<0.001
Soil Organic carbon (%)	1.52±0.47a	1.16±0.51a	2.34±0.98a	1.68 ± 0.85	22.751	<0.001
Soil Organic matter (%)	2.58±0.82a	2.03±0.89a	4.23±1.73b	2.96 ± 1.54	26.675	<0.001
Carbon: nitrogen ratio	15.0±13.59b	11.66±10.99ab	9.43±3.59a	12.07±10.49	2.242	0.112

[a] Samples size (n) for each site: 30; [b] Bold number indicates significant difference among the mean ±Standard deviation

in nutrient rich agricultural land. As agricultural land was an open area, received full sunlight and nutrient as well as less biotic interference causes guerilla ramets of *Centella* spreads horizontally rapidly with increased spacer i. e stolon length. *Trifolium repens* showed clonal foraging response to mild shading, with increased stolon lengths relative to no shading; however, with deep shading where growth was greatly reduced, this response was no longer shown and only short internodes were produced (Thompson 1993). Lowest ramet density of *C.asiatica* in agricultural land (Table1) could be due to periodic disturbance during agricultural practices. There appears trade off between densities of ramets and number of flower per inflorescence. The number of flower per inflorescence was highest at agricultural land where ramet density was the lowest. At this site more than 90% ramets bore flower but at non grazing land about 40% of the total ramets bore flowers. Failure of large proportion of ramets to bear flower at non grazing land may be due to density dependent factors such as competition for resources (eg. nitrogen), space as well as light factor. The lowest number of flowers per inflorescence in non-grazing lands may also be due to light factor. Dense growth of associated species could also be less favourable for the production of flowers. The plants of non grazing land tended to invest less in sexual fecundity and more in traits ensuring vegetative offshoots. Different patterns of ramet recruitment and growth in different habitats also underlie variation in population growth rates in other perennial herbs (Tictin and Nantel 2004).

Morphological Variation

Land use history had significant influence on the morphological characters such as petiole length and SLA but no effect ($p=0.389$) on leaf dry mass (Table 1). The shape of leaf did not vary with land uses. There was significant difference ($p>0.001$) in SLA among three populations. The variation in SLA may be due to different light intensity (Hughes and Cockshull 1972, Nederhoff et al. 1972 Heuvelink and Marcelis 1996) or may due to variation of leaf nutrient. The highest SLA value (401.45 $cm^2\ g^{-1}$) of *C. asiatica* in non grazing land may be due to reduction in available light to the leaves when the plant density was high. A positive effect of plant density on SLA has been found in other crops, e.g. potato (Vos 1995), tomato (Heuvelink and Marcelis 1996) and *Impatiens capensis* (Maliakal et al. 1999); Plants grown in high light generally have thick leaves with low SLA (Bjorkman 1981).Average SLA of *Centella* lies near the median range (14 to 150 g m^{-2}) of global data set for 2548 species compiled by Wright et al. (2004).

There was no consistent pattern of variation in morphological characters among the three sites. Significant difference in petiole length ($P<0.001$) among sites could be due to different light conditions. Petiole length of *Centella asiatica* in non grazing land was the longest (Table 1). Long petiole raises the leaf lamina and enables the plant to receive adequate light when density and height of associated species is high. This is a common strategy of light demanding herbaceous species. Shortest petiole length of *C. asiatica* at agricultural land was due to less density of associated

species and more open area as it received sufficient amount of light. There is no need to develop long petiole for the plant. Plasticity in lamina and petiole form occurs both between and within plants in response to contrasting exposure to light (Niklas 1999, Niinemets and Fleck 2002).

All the morphological characters measured in this study depend on the size of leaves and number of flowers per inflorescence. Since size of organs is much plastic than the number of organs (Harper 1977) most morphological characters showed variation among the sites .Plastic responses of morphological characters to environmental factors such as light and nutrient availabilities are the major cause of intraspecific variation in clonal traits (Birch and Hutchings 1994) .

Nutrient Status

Land use history and ramet density of *Centella asiatica* had significant influence ($p<0.001$) on the leaf N content of this species. Leaf N content increased with decreasing ramet density at these three sites. The agricultural land with highest leaf N content had lowest ramet density, highest number of flower per inflorescence and relatively high soil N content, where as opposite were the cases at non grazing land. Lowest value of leaf N content (0.67%) of *C. asiatica* at non grazing land was may be due to shaded site. Shaded leaves have a lower N concentration than exposed leaves (Lusk 2002). Plant density did not vary significantly with soil nitrogen ($p>0.05$).It appears that high ramet density and low soil N content may be responsible for low leaf N content and high proportion of flowerless ramets at non-grazing land. Similarly, sexual reproductive effort was highest at N rich site, i.e. the agricultural land.

Leaf N of agricultural land was higher than other sites as soil N content was also higher at this site (Table 2). That might also be due to less biotic competition for nutrient resorption and periodic input of fertilizer at agricultural land. Though leaf N appeared to be independent of soil N (correlations, $p>0.05$), the leaf N content of *C. asiatica* was 4-5 times higher than soil N content. This could be possible because the performance of a ramet of a physiologically well integrated plant is not governed by local conditions and ramet can exceed patch nutrient availability (Niva et al. 2003).

Average leaf N of *Centella asiatica* was less than the range of values for tropical grasses (0.9–2.3%, Jha 2003) as well as the median range (0.2-6.4%) of the global data set for 2548 species (Wright et al. 2004).

Land use history had significant impact on soil N and soil organic carbon (OC) (Table 2). High soil OC in agricultural land could be due to addition of organic fertilizer during cropping period. Low OC in soils of non grazing land could be the result of continuous utilization of resources of soil by plant and not further addition of fertilizer as well. Besides these, removal of plant biomass by human activities like cutting of grasses also the cause of low soil nutrient at non grazing land. Likewise low OC in soils of grazing land may be due to removal of above ground biomass by livestock, which removed plant biomass and dilutes the soil OC. Soil OM of present sites was slightly higher than the values (2.32%) reported for waste lands of riverside in the same valley (Koju 2005). Soil OC of the present study sites lies within average values (1.34-3.35%) reported for soil of tropical zone of eastern Nepal (Jha 2003).

Average soil N content of the study sites was lower than the global average of soil N content which is 2g/kg (0.2%) (Larcher 1995), while the value was close to soil N content of warmer climates (tropical and subtropical) where soil N is generally >0.1(e.g. Banarjee et al. 1989, Paudel and Sah 2003, Jha 2003).

CONCLUSIONS

In conclusion, land uses had significant effect on ramet density, petiole lengths, number of flower per inflorescence, leaf N, soil N, OC, OM and stolon length but had no effect ($p=0.389$) on dry mass of leaf. Long petiole and less number of flower at non grazing land and short petiole with more number of flower per inflorescence at agricultural land of *C. asiatica* indicate light demanding nature of the plant. Leaf petiole length and number of flowers per inflorescence showed plastic responses, which appeared to be governed by light availability and is the major cause of population variation in *C.asiatica* plant.

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Quantitative Analysis of Secondary Metabolites in *Centella asiatica* Grown Under Different Habitats

by Anjana Devkota[1*], S. Dall'Acqua[2], G. Innocenti[2] and P.K. Jha[1]

[1] *Central Department of Botany, Tribhuvan University, Kathmandu, Nepal*
[2] *Department of Pharmaceutical Sciences, University of Padova, Padova, Italy*

Key words: Asiaticoside; Asiatic acid; *Centella asiatica*; Terpenes; HPLC–DAD–ELSD; Madecassic acid

Centella asiatica (L) Urban is a very well known medicinal plant, widely used in the traditional medicine, pharmacy, and nutraceutical industry. Active constituents of this plant are triterpenes. Its main constituents, used nowadays as the markers for the assessment of the quality of drugs, are asiaticoside, madecassic acid, and asiatic acid. *Centella asiatica* occurs in Nepal and, if cultivated and harvested, might become a valuable resource. Thus, the major objective of this work was to determine the content of triterpenes: asiaticoside, madecassic acid and asiatic acid in the plants growing at different altitudes by HPLC–DAD–ELSD.

Centella asiatica (L) Urban jest znaną rośliną leczniczą powszechnie używaną w tradycyjnej medycynie, farmacji i w przemyśle odżywek. Aktywnymi składnikami tej rośliny są triterpeny, a ich głównymi składnikami, używanymi obecnie jako markery do oceny jakości leków, są: asiaticoside, madecassic acid i kwas azjatycki. *Centella asiatica* występuje w Nepalu i jej hodowla może stanowić wartościowe bogactwo naturalne. Celem pracy jest oznaczenie wymienionych składników triterpenu w roślinach rosnących na różnych wysokościach. Do oznaczania zastosowano wysokosprawną chromatografię cieczową z detektorami: evaporative light scattering i diode array detector.

* Corresponding author. E-mail: devkotaa@hotmail.com, devkotaa@gmail.com; Fax: 977-1- 4333515

Centella asiatica (L) Urb. belongs to Apiaceae family. It is commonly named Gotukola (called *ghod tapre* in Nepali) and occurs in swampy areas of Nepal, India, Sri Lanka, Africa, Australia, and other tropical and sub-tropical regions. It is an ethnomedicinal plant used in different continents by diverse ancient cultures and tribal groups. The plant has been also used in the traditional Indian and Chinese medicines for the treatment of such diseases as leprosy and psoriasis, for healing wounds and burns, and against insanity [1, 2]. Plant extracts are often present in memory-enhancing tonics and used for the treatment of mental and stress-related disorders [3, 4]. Clinical trials have shown that it might be helpful in the chronic venous insufficiency [5]. Main active components of *C. asiatica* are glycosides (asiaticoside and madecassoside) and their genins (asiatic and madecassic acids) (Fig. 1); they are used for standardization of this species as described in the European Pharmacopoeia (2000). Triterpenoid derivatives have been identified in *Centella asiatica* in several studies using TLC coupled with high-speed counter-current chromatography [6, 7]. In this study we have evaluated the content of triterpene in *Centella asiatica* growing in different parts of Nepal. Due to the poor UV absorption of triterpenes, the ELSD detector was used.

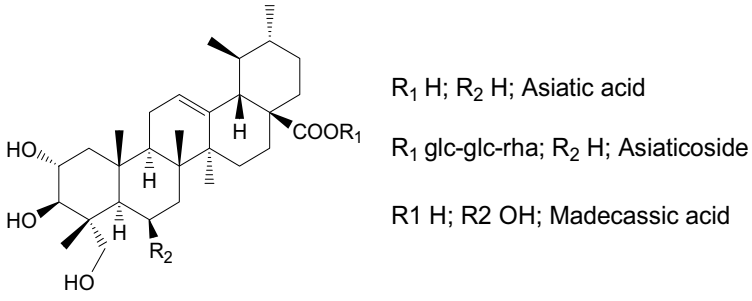

Figure 1. Chemical structure of triterpenes from *C. asiatica* (L) Urban

EXPERIMENTAL

Materials

Eight samples of *Centella asiatica* were collected in August 2007 in different habitats of Nepal, *i.e.* samples CA 20GL in Chitwan, CA 60 GL in Surkhet, CA 85GL in Kaski, CA 72GL in Lamjung, CA 13GL in Kathmandu, CA 180GL in Dhankuta (Pakhribas), CA 200GL in Dhankuta(Hille), and CA 22GL in Tehrathum. All samples were shade dried. Herbaria of voucher specimens were prepared according to the standard methods.

Chemicals and reagents

HPLC grade acetonitrile, methanol, and formic acid were purchased from Carlo Erba, Italy. HPLC grade water was prepared by filtering nanopure water through a 45 μm membrane filter (MilliQ).

Reference sample

Asiaticoside, asiatic acid, and madecassic acid were obtained from a standarized *Centella asiatica* extract kindly gifted by Indena s.p.a (Milan, Italy).

Extraction of plant sample

100 mg of ground plant material (whole plant parts) were placed in 15 mL-in-volume falcon tubes (screw-capped polypropylene centrifuge tubes) and extracted three times with 5.0 mL of methanol by sonication. The extract was centrifuged for 5 min at 3000 rpm. The supernatants were combined, pipetted into a 25 mL volumetric flask, diluted to the final volume with methanol, and mixed thoroughly. All samples were filtered through a 0.45 μPTFE syringe filter prior to the injection into the HPLC system.

Calibration plots

30 mg of reference samples were placed in a 25 mL volumetric flask and diluted with methanol (stock solution). Lower concentrations for calibration were obtained by diluting the stock solution with methanol.

HPLC conditions

An Agilent 1100 series liquid chromatograph equipped with an Agilent 1100 Diode Array detector and an Evaporative Light Scattering Detector Sedex LT60 served for HPLC measurements. A Merk C–18 reverse phase column (250 × 4.6 mm 5 & micro) was used as a stationary phase. Injection volume was 20 μL. All samples were injected three times. For the sample analyses, gradient elution with the following eluents was applied: A – Acetonitrile, B – Methanol, C – Water with 0.1% HCOOH. Details of gradient elution are given in Table 1. Flow rate was set at 1 mL min^{-1}. For a comparison of results, detection was performed also at UV wavelengths 330 nm, 254 nm, and 206 nm using a diode array detector (DAD) instrument. Evaporative light scattering (ELSD) detector temperature was 50°. Nitrogen pressure was set at 2.2 bars. Calibration plot was obtained using the mixture containing standard compounds: asiaticoside, madecassic acid, and asiatic acid at known concentration. Calibration data for the analysed compounds are presented in Table 2.

Table 1. A scheme of gradient elution used in HPLC analyses

Time, min	Acetonitrile (A) %	Methanol (B) %	Water with 0.1% HCOOH
0	10	2	88
10	26	4	70
22	25	5	70
27	30	0	70

Table 2. Calibration data for the analysed compounds

Compound	Regression equation (X axis μg mL^{-1} *vs* area Y axis)	Correlation coefficient, r	Limit of detection, μg mL^{-1} LOD	Linearity range, μg mL^{-1}
Asiaticoside	y = 19493X – 191278	0.9994	13.72	678.0–13.6
Madecassic acid	y = 33930X – 181679	0.9998	7.3	510.6–10.2
Asiatic acid	y = 27491X – 193249	0.9998	6.35	560.8–11.2

RESULTS AND DISCUSSION

The HPLC–ELSD chromatograms of the extracts of *Centella asiatica* collected in different habitats and analysed under gradient elution conditions are shown in Figures 2, 3.

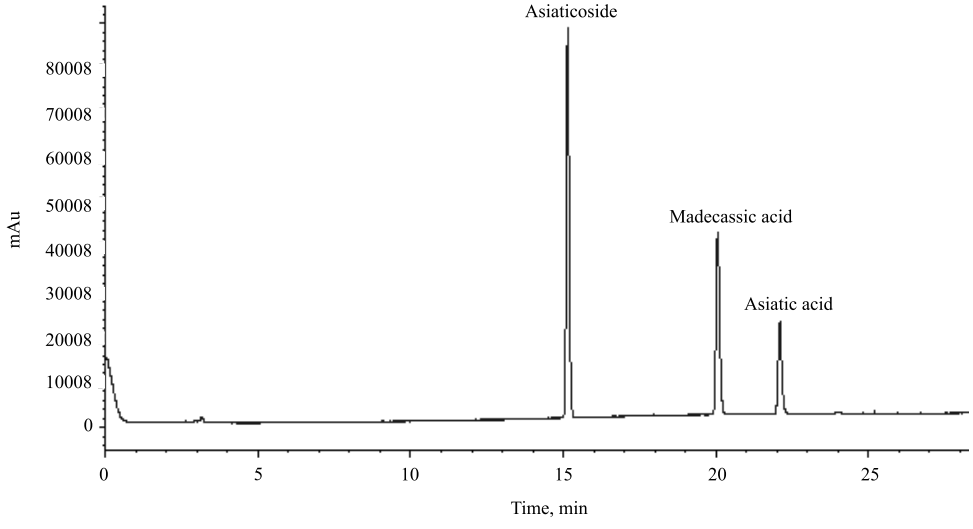

Figure 2. HPLC–ELSD chromatogram of the higher concentration standard mixture of terpenes of *Centella asiatica* (L) Urban

Triterpenes could be preferably detected with ELSD detector rather than with the DAD–UV one because of their poor absorption in the UV–VIS range. For example, asiaticoside present in the standard solution at 13.6 μg mL^{-1} concentration could not be quantified with UV–DAD, but could be easily quantified with ELSD. In addition, applying the latter detection mode, the chromatograms did not need base-line correc-

tion (subtraction of a blank signal in the UV–DAD mode) since the solvent was removed before detection. On the contrary, DAD detection provided the UV spectrum and thus could be very useful for peak identification. For example, the peak measured after 14 min was assigned to phenol derivatives due to its characteristic UV absorption (Fig 3).

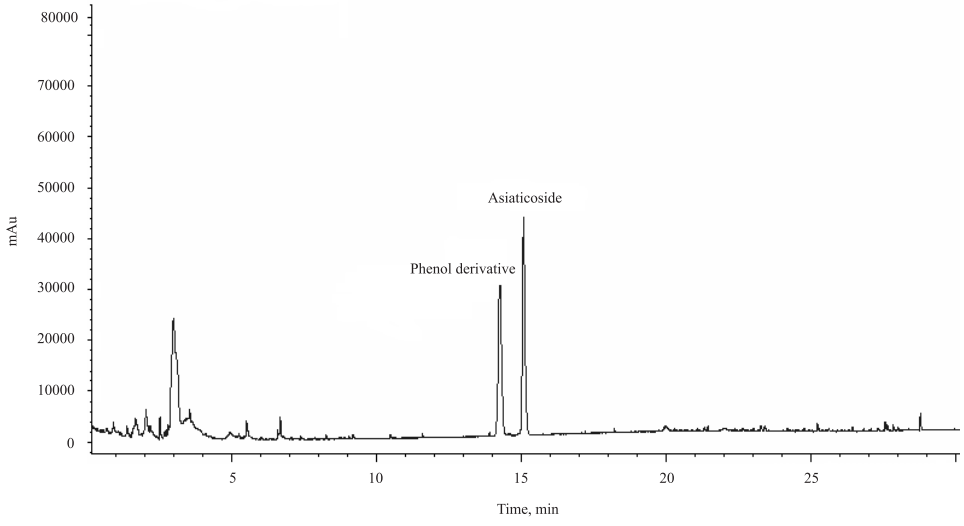

Figure 3. HPLC–DAD (215 nm) chromatogram of one of the analyzed samples (200 m). The peak at *ca* 14 min could be assigned to phenolic derivatives due to its UV spectrum

The results showed that the composition of the analysed specimens and the contents of particular compounds varied among the collected samples (Tab. 3), which may be due to the place of origin of the plant material (differences in environmental conditions) or to different plants' genotypes. Comparative study on 10 ecotypes from different regions of India showed the correlation between the genomic diversity and asiaticoside content [8]; the highest content of asiaticoside was 0.114% in dry mass. The highest total triterpenoid content (1.08%) was observed in 20 GL sample collected at 200 m above the sea level, and the lowest content (0.01%) was found in 22 GL sample collected at 2296 m above the sea level. It has been shown that the concentration of asiaticoside in *C. asiatica* leaves from Nepal is 2–4 times higher than that found in the plant from India [9]. It also seems that the content of these compounds gradually decrease with the increase of altitude. These results have shown that all analyzed sample contain small amounts of triterpenoids. Indeed, a maximum of 4.24% of the total triterpenoid content in dry mass was found in commercial *C. asiatica* plant material purchased from Frontier (Norway, IA, USA) [10]. According to the European Pharmacopoeia (2000) [11], the total triterpenoid content must exceed 6%

in dry mass. In our study, all samples did not fulfil this requirement. However, significant differences in the content of active constituents were observed between the samples of *C. asiatica* originating from different countries, such as India and Madagascar [8, 9]. Indeed, *C. asiatica* grown in Madagascar has been found to accumulate the highest level of asiaticoside [9, 12]. It has been shown to accumulate high concentrations of asiaticoside and madecassic acid and lower of asiatic acid. It has been reported that the lower molecular weight compounds are usually present in plant tissue at relatively low concentrations compared to these of larger polymers [13]. Higher concentrations of asiaticoside and madeccasic acid in *Centella asiatica* samples were confirmed in this study.

Table 3. Concentration (%) of triterpenes determined in *Centella asiatica* samples; LOD – Limit of detection

S.N.	Districts	Altitude, m	Sample no.	Asiaticoside	Madecassic acid	Asiatic acid	Total
1.	Chitwan	200	20 GL	0.53 ± 0.003	0.020 ± 0.001	0.53 ± 0.003	1.08 ± 0.007
2.	Surkhet	600	60 GL	0.49 ± 0.005	0.015 ± 0.001	0.36 ± 0.003	0.865 ± 0.009
3.	Kaski	850	85 GL	0.50 ± 0.002	0.012 ± 0.003	0.11 ± 0.001	0.622 ± 0.006
4.	Lamjung	720	72 GL	0.21 ± 0.03	0.017 ± 0.001	0.21 ± 0.03	0.437 ± 0.006
5.	Kathmandu	1350	13 GL	0.18 ± 0.01	0.015 ± 0.001	< LOD	0.195 ± 0.011
6	Dhankuta (Pakhribas)	2000	200 GL	0.21 ± 0.01	< LOD	< LOD	0.21 ± 0.01
7.	Dhankuta (Hille)	1800	180 GL	0.24 ± 0.01	< LOD	< LOD	0.24 ± 0.01
8.	Terhathum	2296	22 GL	0.010 ± 0.003	< LOD	< LOD	0.01 ± 0.003

CONCLUSIONS

DAD and ELSD detectors were used for quantification of the analytes. ELSD was found to be a good tool for the quantitative determination of triterpenes. The content of triterpenes in the investigated plant varied depending on the environmental conditions, especially the altitude. For this reason, further phytochemical investigations of the triterpene content with respect to different environmental conditions are needed. These findings would be valuable for the evaluation of the best conditions

for cultivation or collection of this medicinal plant. By analysing the secondary metabolites in *C. asiatica* by the proposed technique, we are able to select the appropriate habitat and high yield plant varieties for their further cultivation and conservation.

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Ethnomedicinal Uses of *Centella asiatica* in Gorkha District Nepal

Anjana Devkota[1]
Pramod Kumar Jha[2]

Abstract

Centella asiatica is an important non timber plant species and has medicinal significance It is distributed on moist, tropical to temperate belts of Nepal up to an altitude of 2200m.. It has ethnomedicinal values, and is being used since ancient time especially in rural society. In order to understand its medicinal values in rural society, some accessible parts of Gorkha district was selected. We collected field based information by direct questionnaire survey method to explore on how the local people are using this plant for medicinal purpose. It was observed that, Centella asiaitca has been used against fever, high blood pressure, headache, as memory enhancing, and healing wound and was not collected commercially.

Keywords: Population, Centella asiatica, Ethnomedicnal uses.

Introduction

The Nepal Himalayas are rich pocket of medicinal plants. The recorded list of Medicinal and Aromatic Plants found in Nepal is about 1700 species (Baral and Kurmi, 2006).These are the most important source of medicine for local healers in the village, and the basic raw materials for Ayurvedic, Tibetan, Homeopathic and allopathic medicines.

Himalayan mountains are rich in medicinal plants and mountain people have long tradition of using these medicinal plants for their health care (Thomas et al. 2005). Due to socio-economic transformation, urban people rely mostly on modern allopathic medicine but people of rural areas still use selected medicinal plants and formulations prepared from them. When compounded and prescribed appropriately the safety of traditional herbal medication is high due to moderate bio-reactivity of natural plant products (Elvin-Lewis, 2001). There is a long tradition of transferring cultural knowledge of using medicinal plants from generation to generation. In last few decades these ethnomedicinal knowledge have been exploited for preparing modern medicine, especially in search for novel bioactive natural products which can be used for treatment of serious disease. Ethnomedicinal knowledge can provide a lead for this venture (Hamilton 2003).

Centella asiatica (*Hydrocotyle asiatica* (syn.) Ghodtapre; Umbelliferae (Family) is well known for its medicinal value. It grows widely in moist tropical to subtropical belt up to 2200m altitude above mean sea

[1] *Central Department of Botany, Tribhuvan University, Kathmandu,Nepal devkotaa@hotmail.com*
[2] *Central Department of Botany, Tribhuvan University, Kathmandu,Nepal pkjhaprof@gmail.com*

level. Though it grows widely and used extensively by local for several health problems, but is not in trade in Nepal. Ethnomedicinal use of Centella asiatica has been mentioned in 'Plant and People of Nepal (Manandhar 2002), and Genetic Heritage of Medicinal and Aromatic Plants of Nepal Himalayas (Joshi and Joshi; 2001). Although, this species has been listed in Medicinal and Aromatic Plant Database of Nepal (MAPDON) (Shrestha et al. 2000) and A Compendium of Medicinal Plants in Nepal (Baral and Kurmi, 2006), but details of its use have not been mentioned.

In this paper we report ethnomedicinal use of *Centella asiatica* in Gorkha district, Nepal

Materials and Methods

Study area

The study area (84° 28'-85 ° 10' E longitude and 27 ° 48' -28 ° 45' N latitude) lies on in western part of Gorkha district, Nepal. The district comprises of diversity in physiography ranging from about 400m to its south and 8163m above mean sea level to its north face. The district has high plant diversity (including medicinal and aromatic plants) distributed from tropical to alpine belts. The mountainous region (Northern region) of district comprises the most important and scarce medicinal and aromatic plants such as Aconitum spicatum, Swertia chirayita, Cordyceps sinensis . The southern region of district also comprises important medicinal and aromatic plants such as Asparagus racemosus, Tinospora cordifolia, Phyllanthus emblica and Centella asiatica.

From reconnaissance study following sites were selected for the study: Thantipokhari area (Palumtar Village Development Committee (VDC), Barhapirke area (Palumtar VDC), and Badahare danda (Gaikhur VDC). Among three sites grazing pressure was high in former two sites and received full sunlight, while the latter receives no grazing and is shaded . All these sites were near human settlements and received high anthropogenic disturbances.

Table 1. Physical location of study sites

Sites	Elevation(m)	Location
Thantipokhari	543	28°24.69'N; 84°28.39'E
Badedanda	646.2	28°01.398'N;84°34.63'E
Barhapirke	553.5	28°01.968'N; 84 °29.63'E

Species Characters

Centella asiatica (L.) is a perennial creeping herb, heart or kidney shaped leaves emerging alternately in clusters at the nodes. The petiole is usually 5 to 10cm, the lamina is 10mm to 40mm long and 20mm to 40mm, sometimes up to 70mm, wide. The insignificant greenish to pinkish- white flowers are borne in dense umbels (Clusters in which all the flower stalks arise from the same point) on separate stems in the summer. Flowering occurs in May-June. The flowers are pentamerous, generally consist three flowers rarely two or four in an umbel. The fruit (cremocarp) is compressed sideways and measured 0.1-0.2 inch

(3-5mm) long. It is widely distributed from eastern to western Nepal. In Nepal this plant is not in trade, but widely used in ayurvedic formulation

Field Data Collection

Ethnomedicinal Uses

A set of standard questionnaire was prepared. Middle aged and old men and women of study areas were interviewed individually. They were asked about medicinal use of *Centella asiatica*, parts used and mode of collection, dose, way of use, relative effectiveness and local distribution.

Results

Most of the people other than local healers know the usefulness of Centella asiatica. Every old man and woman knows Ghodtapre very well by name .There was no commercial collection of Centella asiatica. In Gorkha district, it was found that people have been using leaves of Centella asiatica to make pickles. Also it has been used against fever, high blood pressure, cough, high uric acid, and blood sugar. Although the whole plant (termed panchanga, in ayurveda) is medicinally important, local people of study site, collect mainly leaves during available season, as it is a perennial plant. The collected plant material is used either in fresh or, dried in shade. The dried plants are stored in air tight bottle or packets and used against uric acid reduction as well as cooling properties. Decoction of leaves is used against conjunctivitis and other eye injury; crushed leaves are mixed in a cup of water with a tablespoon of salt and taken once daily for stomachic, indigestion and flatulence.

Gorkhalis used many other plants like Eclipta prostata, *Cuscuta reflexa, Adhatoda vasica, against cough,* jaundice, and worm. According to respondents, Centella asiatica is only the effective plant used against many health problems. In Palpa district, leaf paste prepared with cow's urine is applied on nose and forehead to cure sinusitis 'Pinas' (Panthi and Chaudhary 2006) and in Lalitpur district the plant has been used as brain tonic as well as against piles (Sharma and Joshi, 2000).

Although *Centella asiatica* was widely used by local communities in study site, this is not a trading item from Nepal, probably due to small size plant and consuming more time for collection. Since most of the research and conservation efforts have been focused on commercially exploited medicinal plants, medicinally effective species such as Centella asiatica has got no priority in this regard. The plant is listed as threatened species (India) by the international organization (IUCN) (Pandey et al.1993) and an endangered species (Singh 1989, Sharma and Kumar 1998). This plant being endangered in India, the potential of exporting this plant from Nepal to India is high as in Nepal this plant is widespread and abundantly found. This species is not currently in trade but this can be a potential source of bioactive natural products.If we are able to bring it in trade that may be supportive for raising economy of the local community. There should be more studies on the potential of exporting this plant and its benefits to Nepal

Conclusion

Centella asiatica is considered to be one of the most effective and multipurpose plants used by rural people. Although the species is found abundantly on moist open area, there is no commercial collection in Nepal.Research and conservation efforts should focus not only on the species in trade but also the other

species like this which are not currently in trade but are considered highly effective in ethno-medicine, and can be a potential trading item.

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Variation in Growth of *Centella asiatica* along Different Soil Composition

Anjana Devkota and Pramod Kumar Jha

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Abstract: The effect of variation in soil composition on growth vigour of *Centella asiatica* (L) Urb. was investigated in greenhouse of Central Department of Botany, Tribhuvan University,Kathmandu. Variation in different growth traits of *Centella asiatica* was investigated using vegetative clone of genome from one population in Kirtipur, Kathmandu, Nepal. We chose soil composition type as the treatment factor to study variation in growth traits as well as to know the best type of composition of soil for cultivation purpose. We raised plants in each of six soil compositional type and examined an array of vegetative traits like: number of leaves, petiole length, specific leaf area, number of primary branches and plant biomass. Most of the observed growth traits demonstrated significant variation in response to soil type. The *C. asiatica* plant can maximize growth and yield in habitat with sandy loam rather than clayey soil.

Key words: *Centella asiatica* % Growth traits % Soil Composition % Variation

INTRODUCTION

Centella asiatica (L.) Urban (Family: Apiaceae) is an important traditional medicinal plant [1]. The plant is native to India, China, Nepal, Indonesia, Sri Lanka, Australia, Madagascar and Southern and Central Africa [2]. It is found throughout India and Nepal in moist places up to an altitude of 2200m (tropical to subtropical region) and also on moist stone wall or other rocky sunny areas. It is a clonal plant colonising early in the abandoned jhum (slash and burn agriculture) [3].The plant can grow in a variety of soils with moist, sandy or clayey loam, rich in humus. Observation of natural populations of *Centella asiatica* indicated extensive variation in its growth and reproductive traits.

Results of an extensive survey of populations of *C. asiatica* indicated significant effects of site on growth traits [4].We investigated the amplitude of variation in growth traits with respect to soil type in controlled environmental conditions and choose soil type as the treatment factor. It is well established that soil texture governs most soil properties [5], including organic matter accumulation [6], retention and release of water [7] and the amounts of nutrients in plant-available forms [8]. We hypothesized that growth traits and yield of *Centella asiatica* varied significantly with soil type; and these variations underlies the adaptation of plant to resource supply. The main objectives of this study were to study variation in growth of *C. asiatica* under different soil composition and to identify soil type for cultivation to obtain high yield. In present paper, we report patterns of variation in growth traits and yield of *C. asiatica* in response to changes in soil composition.

MATERIALS AND METHODS

Treatment Conditions: A pot culture experiment in a completely randomized design, was established in the Botanical Garden, Central Department of Botany(CDB), Tribhuvan University, Kathmandu, (85º17.32'E Long; 27º40.20'NLat,1350m asl), Nepal. The proportions of clay and sand varied by thoroughly mixing the fine soil (0% sand) (collected from agricultural land) with horticultural sand in different proportion. Thus six treatments (composition) reflecting a gradient of decreasing clay contents (%), 100 (S1), 80 (S2), 60(S3), 40 (S4), 20(S5) and 0(S_6) were prepared (Table1). The six soil types analyzed fall under the following textural classes clay, silt, loam soil, sandy loam, sandy soil and sand (Table 1).

Plant Material: Several plant cuttings of randomly sampled individual plants of *C. asiatica* were collected from same population from garden of CDB, TU, Kathmandu. The cuttings of plantlets were more or less uniform size containing four leaved condition; were planted in earthen shallow pots filled with different types of soil (S1 to S6) in green house. Altogether 300 plants;

Corresponding Author: Anjana Devkota, Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Table 1: Soil textural class and characteristics

Soil type	Textural class	Bulk density (g cm^{-3})	Soil N (%)	Organic carbon (%)
S1	Clay (0 % sand)	1.55	0.25	4.5
S2	Silt (20% sand)	1.4	0.22	4.2
S3	Loam soil (40% sand)	1.35	0.19	3.8
S4	Sandy loam (60%sand)	1.05	0.12	3.58
S5	Sandy soil (80% sand)	0.99	0.09	1.31
S6	Sand (100% sand)	0.88	0.008	0.025

fifty plants for each treatment were planted separately for experiment. Planting was done in October 2007 and equal amount of water was provided for irrigation purpose for each treatment. All pots and treatments were rotated each week to counter any positional effects of pots within treatments.

Growth Measurement: Data on yield and morphological traits were recorded in April 2008. Forty plants per replication, selected randomly, were used for the observations. Twenty quantitative traits pertaining to plant morphology and yield were measured.

Ninety mature leaves per treatment were measured for leaf length (LL), leaf width (LW), petiole length (PL), leaf area (LA), dry weight of leaf (LDW) and specific leaf area (SLA). Petiole length, length and width of leaves were measured in fresh leaves. Then these leaves were oven dried (60ºC, 48 h) and mass of each leaf was weighed in electric balance (0.001g). Length and width of leaves were measured and multiplied by conversion factor following Zobel *et al*. (1987) [9] for determination of leaf area. SLA was calculated as the ratio of leaf area and dry mass.

Leaf nitrogen (N) content was determined by modified microKjeldahl method following the procedure described by Horneck and Miller [10]. Leaf N content was determined in twenty samples from each treatment. Chlorophyll a, Chlorophyll b and total chlorophyll content was determined following the method of Arnon (1949) [11] in five samples from each replication.

Number of nodes (NND) occurring along each primary branch were noted. Internodal lengths (IND) were also measured on primary branches arising from mature rosettes. The diameter of a mature leaf rosette (DR) indicated its spread and number of leaves (NLN) and primary branches (NBN) arising from it was also scored.

Inflorescences were measured for flower pedicel length (FPL) and total number of flowers per mature rosette. Fresh (FHY) and dry (DHY) herb yields per replication were obtained after harvest and moisture content (MC) calculated.

Soil Analysis: Air-dried soil samples (n =5), brought separately for each soil type for analysis. Organic C was determined by the Walkley Black rapid titration method and total N by the micro-Kjeldahl method [12].

Statistical Analysis: The significance of the difference between the mean of measured attributes among the soil type was analyzed by one way analysis of variance (ANOVA).The amount of variation in the parameters in response to the treatments was assessed by calculating the coefficient of variation (CV) computed as the standard deviation of the mean values in each of the six soil types divided by the overall mean of the six treatment means [13].The treatment types were also compared by multiple range tests (Duncan Homogeneity test). Statistical Package for Social Science (SPSS, version, 11.5, 2002) was used for all statistical analysis.

RESULTS AND DISCUSSION

Soil Texture and Fertility: In the present study, clay content was significantly related to organic C (r^2= 0.88, P <0.001) and total N (r^2 = 0.85, P <0.001). Studies have reported that, in soils with relatively high clay content, the stabilizing complexes are resistant to microbiological decomposition [14]. Furthermore, anaerobic conditions in fine-textured soils can increase denitrification losses [8, 15] and reduce mineralization of organic N [16]. Therefore, despite high organic C and total N, the amount of plant-available N would be lower in clay-rich soils. Sand would also be low in fertility because of small total N. Thus, the sandy loam (S4) would be expected to be the most fertile soil.

Morphological Traits and Dry Mass: All the measured traits of leaves varied significantly with soil type, however, the differences was only marginal for leaf dry weight and leaf area (Table 2). Among the leaf traits, the extent of variation was the highest in leaf dry weight (CV=1.48, Table 2) and lowest in total chlorophyll content (CV=0.043).Average number of leaves was 4.15 per ramet.

Table 2: Leaf characters of *Centella asiatica* in different soil textural type. For each parameter significant difference between mean among different sites are indicated by different letters (Duncan homogeneity test, á = 0.05). F and P values were obtained by one way analysis of variance (ANOVA)

Attributes[a]	0%sand (S1)	20%sand(S2)	40% sand(S3)	60%sand(S4)	80%sand(S5)	100%sand(S6)	Mean	CV	F value	P value[b]
Petiole length (cm)*	11.51±4.77bc	12.54±6.83c	12.69±4.95c	12.38±3.57c	9.16±2.88ab	8.22±2.52a	10.9±4.62	0.42	3.604	0.005
Leaf length(cm)*	2.36±0.26	3.21±1.02	2.62±0.6	2.52±0.33	2.49±0.34	2.26±0.38	2.56±0.61	0.23	6.22	0.000
Leaf width(cm)	4.36±0.47a	4.41±3.23b	4.4±0.93a	6.13±0.6a	4.01±0.64a	4.03±0.75a	4.6±1.53	0.33	4.64	0.001
Dry wt of leaf(g)*	0.081±0.03a	0.083±0.041a	0.07±0.045a	0.05±0.02	0.11±0.24b	0.09±0.09a	0.09±0.12	1.33	2.52	0.034
Leaf Area(cm^2)*	10.16±2.03a	18.73±20ab	19.84±5.43a	14.63±2.94	21.23±7.52b	13.62±20.17a	16.81±24.91	1.48	2.26	0.054
SLA(cm^2g)*	127.13±63.19	234.86±192.52c	283.58±70.72ab	292.2±43.69	193.55±34.87	151.33±56.34a	186.99±96.36	0.51	3.68	0.004
Leaf N (%) ^	1.69±0.31	2.11± 0.18	2.1±0.107	2.16±0.0.195	1.65±0.12	1.26±0.18	1.26±0.057	0.4	67.55	0.000

[a] Sample size(n) for each treatment: *n=90 [#] n=5 ^ n=20;b Bold number indicates significant difference among the mean, ± Standard deviation, CV= Coefficient of Variance

Table 3: Growth traits and yield of *Centella asiatica* in different soil textural group. For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, á = 0.05). F and P values were obtained by one way analysis of variance (ANOVA)

Attributes[a]	0%sand(S1)	20%sand(S2)	40% sand(S3)	60%sand(S4)	80%sand(S5)	100%sand(S6)	Mean	CV	F value	P value[b]
Rosette diam(cm)[$]	12.4±2.13d	15.28±3.33c	18.18±3.15d	19.52±2.29c	13.25±1.86b	10.5±3.03a	14.62±3.62	0.24	17.19	0.000
No. of leaves[$]	3.66±.5a	4.31±.28a	3.25±0.55a	5.29±0.8a	4.65±0.52a	3.65±0.24a	4.25±1.54	0.36	1.499	0.197
Number of pri.branch[$]	2.73±1.33b	3±0.96b	2.87±1.25b	2.76±1b	2.7±0.92b	1.85±0.7a	2.62±1.09	0.41	2.86	0.018
Number of nodes[$]	4.66±3.24a	3.43±1.26a	4.93±3.43a	4.94±2.16a	5.05±2.01a	4.7±1.62a	4.64±2.37	0.51	1.052	0.392
Length of internode(cm) [$]	11.81±1.69a	22.14±45.04a	12.28±1.93a	11.77±1.44a	10.79±1.89a	9.32±1.25a	12.79±17.74	0.13	1.12	0.357
Peduncle length(cm) [$]	1.85±4.52a	1.92±0.795a	1.89±0.54a	1.87±0.74a	1.85±0.58a	1.52±0.51a	1.8±00.6a	0.33	0.393	0.393
Number of flower per node[$]	9.1±2.18a	12.26±3.95ab	18.42±4.7b	21±4.13b	12.7±3.26ab	15.16±3.88ab	15.17±3.89	0.25	1.75	0.131
Moisture content of plant[$]	73.45±1.2a	72.61±3.25b	74.42±1.12a	71.55±2.21ab	64.7±1.2b3	43.75±1.2b	66.74±2.15	0.03	12.36	0.000

[a] Sample size(n) for each treatment: [$] n=40 ;b Bold number indicates significant difference among the mean, ± Standard deviation, CV=Coefficient of Variance

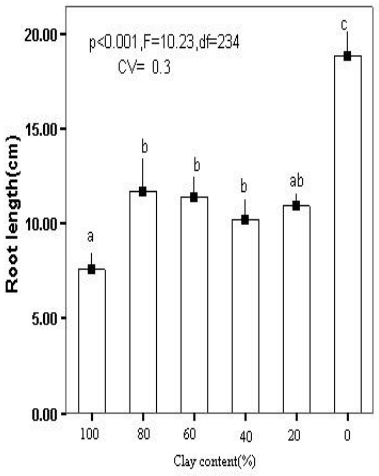

Fig. 1: Effect of soil texture on root length of individual plant

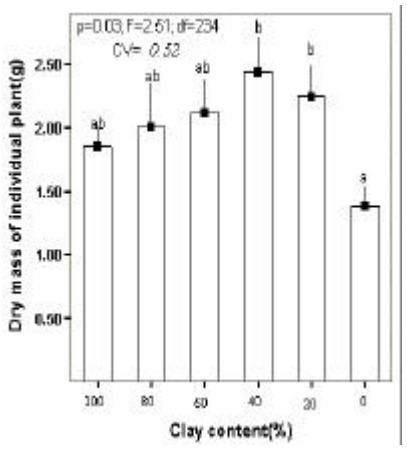

Fig. 2: Effect of soil texture on dry mass of individual plant

There was no significant difference in leaf number among the treatments (Table 3). Diameter of rosette and root length of plants varied significantly (p<0.001) among treatments with longest (18.9cm) root found in S6 type of soil (i.e. pure sand; Fig1). In present study, plants had relatively low SLA if grown on sand as well as on soils with excessively high clay content. Plants grown at low nutrient availability show a decrease in SLA [17, 18] partly as a result of the accumulation of non-structural carbohydrates or secondary compounds such as lignin or other phenolics [19]. Greater SLA values indicate more leaf surface per unit biomass and, thus, more area available for photosynthesis [20], which compensates for lower leaf area in plants, as found in this study for plants grown in 40% clay (S4).

Growth of root was also affected significantly with soil type. Under extreme water stress; the roots more easily penetrated the loose-dry soil treatment, as supported by the observed root depth. More macropores allowed root penetration and soil particles were more easily pushed aside in the loose treatment due to higher porosity, possibly providing greater access to the little water available and promoting production as a result. Low nutrient supply results in an increased root length

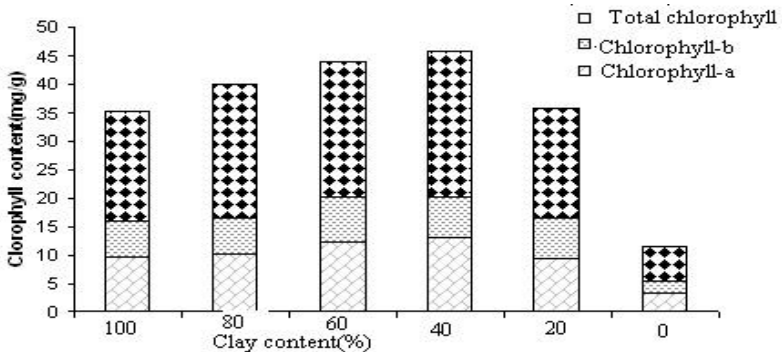

Fig. 3: Effect of soil texture on chlorophyll content of leaf of *C. asiatica*

ratio [21, 22]. Thus the longest root length in sand was attributed by low nutrient and water supply. Under arid and stressed condition, overall plant growth was reduced as a result of both biochemical disruptions and reduced cell enlargement, which in turn led to reduced leaf expansion and total leaf area and therefore reduced whole plant photosynthesis. That is the reason for low value of growth trait data and low yield in pure sand.

Further, inadequate contact of roots with the soil in sands could limit the uptake of water and nutrients, which in turn, appears to reduce the growth rates in *C. asiatica*. Sultan and Bazzaz [23, 24, 25] studied variation in growth traits in *Polygonum persicaria* in response to light, moisture and nutrient content of soil and they found marked morphological variation in leaf, stem, root and fruit and in structures related to reproductive support following changes in soil moisture [24].

Dry mass of plant was differed significantly among the soil type (p=0.035). It was higher in sandy loam (S4 type) of soil (Fig 2).Sandy loam soil with medium bulk density may have facilitated high biomass production. Compared to low and high bulk densities, the medium bulk density may provide longer retention of water in the soil and increase available water to plant due to the higher proportion of mesopores. Uptake of water and nutrients may also be improved by better root-soil contact [26]. In contrast to that soil having high (S1, 100% clay) and very low bulk density did not favor the growth of plants resulting low yield. Siemens *et al* [27] reported that limited resources (e.g. soil nutrients, water, air) can directly inhibit the rate of growth. According to Bazzaz [28], availability of soil resources, especially nutrients, critically influenced plant growth. Hence, comparatively low biomass exhibited on clay-rich soils (S1 treatment) suggests that *C. asiatica* had slower growth rates under soil having low available nutrient and air due to compactness of soil particles. i.e high bulk density. Further, inadequate contact of roots with soil in coarse textured soil i.e. in sand; could limit the uptake of water and nutrients [29], which in turn, appears to reduce the growth rate and yield of *C. asiatica*. On the other hand, comparatively high yield and growth vigour observed in sandy loam (S2, S3 and S4) type treatments would enable the plant to pre-empt growth resources.

Leaf Nutrient and Chlorophyll Content: Leaf N content ranged from 1.46 to 2.11 %(average 1.26%) (Table 2). Soil textural type had significant influence (p<0.001) on the leaf N of *Centella asiatica*. Chlorophyll a content ranged from 3.25 at soil with no clay (S6) type to 13.17mg/g at 40% clay (S4 type) soil (average 8.37 mg/g) (Fig 3).Chlorophyll b content also ranged from 2.01 at S6 type to 7.88 mg/g at 60 % clay (S3 type) soil. There was significant difference (p<0.001) in chlorophyll a and b content among the treatments. Leaf N and chlorophyll content was the least in plants grown in pure sand (Table 2). Lowest value of leaf N (1.26%) and chlorophyll (6.23mg/g) content of *C. asiatica* grown in pure sand was due to poor nutrient (0.001% N) and less moisture content in that soil. The decrease of chlorophyll content in plants growing in sand may be associated with and most probably related to the decrease of plants water content (43.75%). The decrease of chlorophyll a content is highly related to the decrease of water content in plant leaves [30, 31]. Higher value of leaf N (2.16%) and total chlorophyll content (25.48mg/g; Fig 3) of *C. asiatica* plant grown in 40% clay was due to sufficient amount of air, nutrient and water.

In conclusion there was significant variation in many vegetative traits and yield of *C. asiatica* along different soil compositional type. The results also suggest that *C. asiatica* can maximize growth and yield in habitat with sandy loam type of soil rather than clay. This information can be used in planning cultivation of the plant.

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Centella asiatica (L.) urban from Nepal: Quali-quantitative analysis of samples from several sites, and selection of high terpene containing populations for cultivation

Anjana Devkota [a], Stefano Dall'Acqua [b,*], Stefano Comai [b], Gabbriella Innocenti [b], Pramod Kumar Jha [a]

[a] Central Department of Botany, Tribhuvan University, Kathmandu, Nepal
[b] Department of Pharmaceutical Sciences, University of Padova, Via Marzolo 5, 35131 Padova, Italy

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ABSTRACT

Centella asiatica (L.) Urban is widely used in traditional medicine in many countries and in the formulation of drugs and cosmetics, and is therefore suitable as a trade item for the development of medicinal plants for the population of Nepal. The aim of this work was to select plant populations of *C. asiatica* with high contents of secondary metabolites growing in various localities in Nepal, and to enhance knowledge of the cultivation of this plant. Quali-quantitative analysis of bioactive triterpenes (asiaticoside and asiatic acid) and phenol derivatives (flavonoids and caffeoyl esters) was performed by HPLC-DAD-ELSD. The highest quantities of triterpenes and phenols were found in samples from the Gorkha and Chitwan districts. Regarding cultivated plants, soil fertilisation is critical, since over-rich soils affect secondary metabolite content. Plants growing in sand-rich soils produce more terpenes. This work provides indications on how to select high-terpene producing germplasm and recommendations for plant cultivation.

1. Introduction

Centella asiatica (L.) is a stoloniferous perennial herb, commonly growing in humid areas in several tropical countries. It is used as a remedy for many diseases in the folk medicine of several countries (Shrestha and Dhillion, 2003; Mamedov, 2005).

C. asiatica is widespread in both eastern and western areas of Nepal, and is collected by the native people for many traditional medicinal uses. For example, aerial parts are used against fever, to reduce uric acid levels, to treat high blood pressure, and as a memory enhancer (Devkota and Jha, 2008). Literature data on the phytochemistry and pharmacological activity of *C. asiatica* were reviewed in 2007, and research has been reported both *in vitro* and *in vivo* (Jamil et al., 2007). The main active principles of *C. asiatica* are triterpene glycosides and their respective aglycones. However, significant differences in active constituent contents have been observed between samples from several countries, including India (Das and Mallick, 1991) and Madagascar (Rouillard-Guellec et al., 1997). Other published data indicate that *C. asiatica* grown in Madagascar accumulates the highest levels of asiaticoside (Rouillard-Guellec et al., 1997; Randriamampionona et al., 2007).

Extracts and active constituents are found in many herbal drugs and cosmetic preparations worldwide, especially for venous circulation and skin care (Cesarone et al., 2001; Incandela et al., 2001). *C. asiatica* is therefore suitable as a trade item for the development of medicinal plants for the population of Nepal.

* Corresponding author. Tel.: +39 049 8275332; fax: +39 049 8275366.
E-mail address: stefano.dallacqua@unipd.it (S. Dall'Acqua).

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To our knowledge, although no serious efforts have been devoted to planning and organising the cultivation of *C. asiatica* in Nepal, its spontaneous collection and over-exploitation are now widespread, due to high market demand, and threaten spontaneous populations of this important medicinal species. Invasive plant species like *Parthenium hysterophorus* also threaten *C. asiatica* populations.

The main aims of this study were to select good-quality plant samples of *C. asiatica* for further cultivation and conservation, and to find the best environmental conditions yielding plants with high contents of active compounds. For these reasons, we choose to analyse the content of some marker compounds to establish the quality of the plant material. Three classes of compounds were analysed: asiaticoside and asiatic acid were selected as markers for terpene contents, quercetin-3-O-glucuronide, quercetin and kaempferol for flavonoids/and rosmarinic and chlorogenic acids for phenylpropanoid contents.

Samples of cultivated *C. asiatica* were also analysed for further information on its possible cultivation in Nepal.

2. Materials and methods

2.1. Plant material

C. asiatica (L.) plants were collected and shade-dried/in three regions of Nepal: Eastern (86°E long. to 88°15′E long.), Central (83°E long. to 86°E long.) and Western (80°E long. to 83°E long), during May 2007 and 2008. Herbaria of voucher specimens were prepared according to standard methods, and are deposited at Tribhuvan University herbarium (TUCH) (NCA1-CA38).

2.2. Cultivation

The germplasm of wild plants were used to cultivate a small plot in Kathmandu. Planting was carried out in the last week of October 2007 and plants were harvested in May 2008. During cultivation, soil from the garden of the Central Department of Botany, Tribhuvan University (T.U.) was used. Plants were watered when necessary.

2.3. Plant cultivation in various environmental conditions

Experiments to evaluate differing environmental conditions were performed on plantlets in pots of equal size from the botanical garden at Kirtipur, Tribhuvan University. All experiments were carried out in the last week of October and the plants were harvested in May 2008. Environmental conditions tested were: water stress conditions (125%, 100%, 70% and 30%) Field water Capacity (FWC), distribution, light conditions (70% shade, 50% shade and 30% shade), percentages of sand (0, 20, 40, 60, 80, 100%), compost per pot (10, 20 g) and addition of urea. Except for watering experiments, plants were watered equally in all pots as necessary. Except for soil composition experiments, the soil composition used was one part garden soil, one part compost and two parts sand.

2.4. Chemicals and reagents

MilliQ water was from Millipore; HPLC grade acetonitrile, methanol and formic acid were purchased from Carlo Erba, Italy.

2.5. Reference samples

Asiaticoside, kaempferol, quercetin, rosmarinic and chicoric acids were purchased from Phytolab GmbH, Germany. Chlorogenic acid was purchased from Sigma Aldrich. Quercetin 3-O-glucuronide was purified from *C. asiatica* extracts, as described elsewhere (Satake et al., 2007).

Table 1
Concentration ranges and calibration curves for analysed secondary metabolites.

Analyte	Concentration range (μg/mL)	Regression curve[a]	($n = 6$) R^2	LOD (μg/mL)	LOQ (μg/mL)
Asiatic acid	4.25–100.0	$\text{Log } y = 0.608 \text{ Log} x - 4.640$	0.9976	1.04	3.46
Asiaticoside	5.62–140.5	$\text{Log } y = 0.588 \text{ Log} x - 4.220$	0.9989	1.02	3.40
Chicoric acid	0.732–73.17	$y = 0.0127x - 0.0886$	0.9998	0.29	0.97
Chlorogenic acid	0.685–97.53	$y = 0.0168x - 0.2456$	0.9989	0.40	1.33
Rosmarinic acid	0.766–113.0	$y = 0.0147x + 0.1534$	0.9995	0.35	1.17
Quercetin	0.84–84.03	$y = 0.0086x + 0.9724$	0.9982	0.21	0.70
Quercetin 3-O-glucuronide	1.3–130.0	$y = 0.0393x - 0.0249$	0.9983	0.10	0.33
Kaempferol	0.66–65.97	$y = 0.0136x + 0.391$	0.9997	0.19	0.63

[a] x: peak area; y: concentration of analyte (μg/mL).

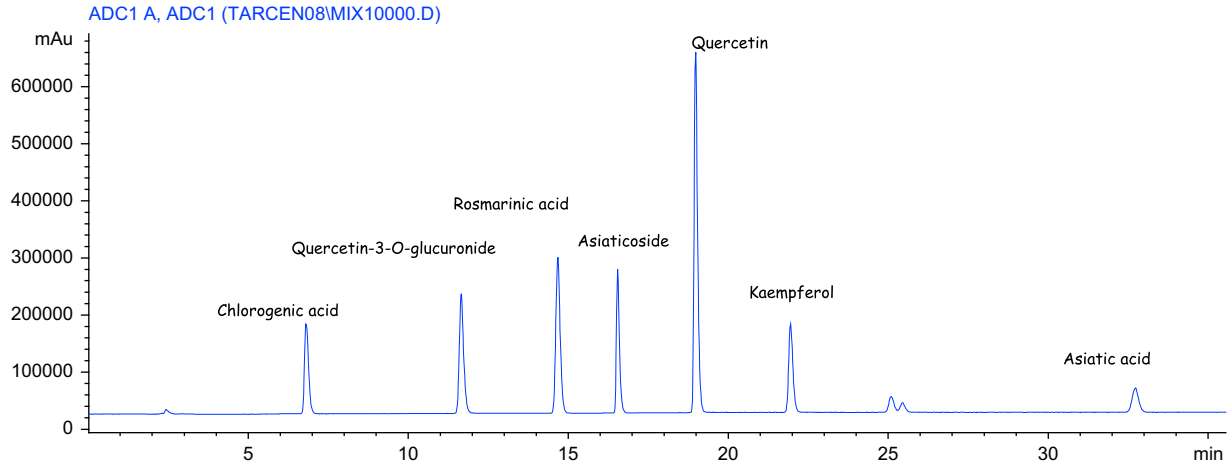

Fig. 1. HPLC-ELSD chromatogram of the standards used for the quantitative determination.

2.6. Extraction of plant samples

Dried aerial parts were ground and 100 mg were placed in a 15-mL falcon/tube (screw-capped polypropylene centrifuge tube) and extracted three times with 5.0 mL of methanol by sonication with Falc Ultrasonic UT460 bath (50 Khz), at room temperature. The extract was centrifuged for 5 min at 3000 rpm and the supernatant was combined in a 50-mL volumetric flask, diluted to final volume with methanol, and mixed thoroughly. All samples were filtered through a 0.45-μm PTFE syringe filter prior to injection in HPLC.

2.7. HPLC conditions

Instrumentation consisted of an Agilent 1100 series liquid chromatograph equipped with an Agilent 1100 Diode Array (DAD) and a SEDEX LT60 Evaporative Light Scattering Detector (ELSD). An Agilent XDB-C-18 reverse phase column (25 × 4.6 mm, 4.6 μm) was used. The gradient elution program, with aqueous formic acid (0.1%) (A) and acetonitrile (B),

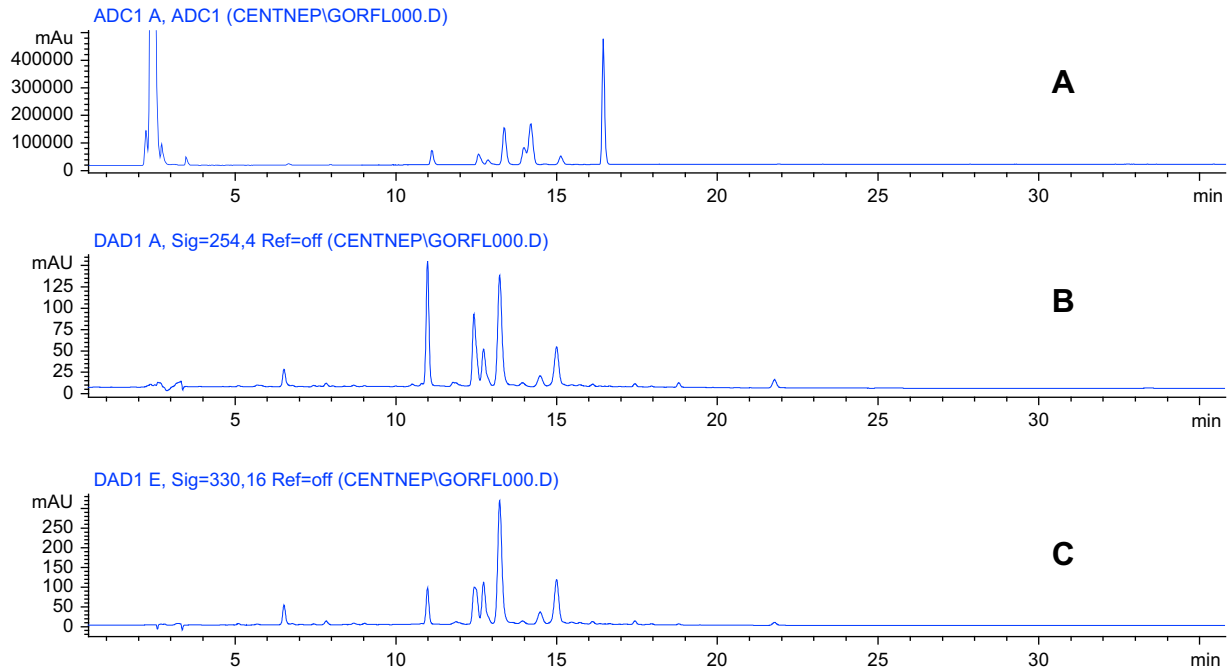

Fig. 2. HPLC chromatographic traces of one selected sample of *C. asiatica* recorded by ELSD (A) and DAD at 254 nm (B) and 330 nm (C), respectively.

Table 2
Amount of selected phytoconstituents in Eastern Nepal wild plants, expressed as % ±SD.

Site (Location)	Altitude (m)	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O-glucuronide	Kaempferol	Quercetin
Ilam (87°90.32′E Long.; 26°72.80′N Lat.)*	1250	1.087 ± 0.045	N.D.	N.D.	N.D.	N.D.	0.343 ± 0.005	0.037 ± 0.002	N.D.
Jhapa (88°3.05′E Long.; 26°31.6′N Lat.)*	90	1.100 ± 0.015	N.D.	N.D.	N.D.	N.D.	0.188 ± 0.005	N.D.	N.D.
Sunsari (87°1.61′E Long.; 26°41.28′N Lat.)*	85	1.532 ± 0.054	N.D.	0.050 ± 0.009	0.141 ± 0.004	0.058 ± 0.002	0.084 ± 0.004	0.370 ± 0.001	0.377 ± 0.004
Sunsari (87°1.61′E Long; 26°41′N Lat.)	85	1.064 ± 0.055	N.D.	0.045 ± 0.009	0.066 ± 0.002	0.053 ± 0.002	0.173 ± 0.004	0.370 ± 0.002	0.371 ± 0.003
Dhankuta (87°3.54′E Long; 26°77.70′N Lat.)*	2000	1.228 ± 0.045	0.332 ± 0.023	0.040 ± 0.003	0.047 ± 0.001	0.056 ± 0.002	0.299 ± 0.002	0.390 ± 0.001	0.390 ± 0.005
Mean		1.202	0.066	0.027	0.056	0.039	0.217	0.239	0.238

N.D. = amount was under LOQ; '*' germplasm used for cultivation.

Table 3
Amount of selected phytoconstituents in Central Nepal wild plants, expressed as % ±SD.

Site (Location)	Altitude (m)	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O-glucuronide	Kaempferol	Quercetin
Makwanpur (85°0.32′E Long; 27°37.5′NLat)*	650	0.622 ± 0.055	N.D.	N.D.	N.D.	0.034 ± 0.001	0.231 ± 0.009	0.321 ± 0.008	N.D.
Chitwan1 (84°3.22′E Long; 27°60.71′NLat)*	200	5.870 ± 0.044	N.D.	0.061 ± 0.002	0.254 ± 0.011	0.171 ± 0.008	0.858 ± 0.023	0.372 ± 0.011	0.373 ± 0.014
Chitwan 2 (84°3.22′E Long; 27°60.71′N Lat)	200	2.638 ± 0.043	N.D.	0.052 ± 0.002	0.171 ± 0.008	0.074 ± 0.002	0.256 ± 0.008	0.384 ± 0.006	0.385 ± 0.014
Chitwan 3 (84°3.22′E Long; 27°60.71′N Lat.)	200	3.912 ± 0.022	N.D.	0.070 ± 0.002	0.351 ± 0.011	0.132 ± 0.06	0.749 ± 0.021	0.391 ± 0.008	0.391 ± 0.003
Kathmandu 1 (85°17.32′E Long; 27°40.20′N Lat.)*	1350	0.571 ± 0.018	N.D.	0.0446 ± 0.001	0.358 ± 0.007	0.213 ± 0.005	1.031 ± 0.016	0.375 ± 0.005	0.376 ± 0.003
Kathmandu 2 (85°17.32′E Long; 27°40.20′N Lat.)	1350	0.529 ± 0.040	N.D.	0.067 ± 0.001	0.318 ± 0.012	0.131 ± 0.004	2.440 ± 0.045	0.352 ± 0.007	0.357 ± 0.009
Kathmandu-3 (85°17.32′E Long.; 27°40.20′N Lat.)	1350	0.550 ± 0.043	N.D.	0.064 ± 0.002	0.446 ± 0.017	0.220 ± 0.008	1.287 ± 0.033	0.397 ± 0.006	0.391 ± 0.008
Kathmandu-4 (85°14.34′E Long; 27°40.54′NLat.)	1400	0.540 ± 0.023	N.D.	0.041 ± 0.001	0.294 ± 0.012	0.196 ± 0.009	2.429 ± 0.023	0.383 ± 0.003	0.380 ± 0.003
Kathmandu-5 (85°14.34′E Long.; 27°40.54′NLat.)	1400	0.504 ± 0.039	N.D.	N.D.	0.375 ± 0.009	0.197 ± 0.004	1.837 ± 0.026	0.387 ± 0.007	0.385 ± 0.005
Kathmandu-6 (85°14.34′E Long.; 27°40.54′N Lat.)	1400	0.526 ± 0.038	0.293 ± 0.015	0.042 ± 0.002	0.303 ± 0.007	0.193 ± 0.006	2.139 ± 0.012	0.390 ± 0.002	0.394 ± 0.001
Lalitpur 1 (85°22.70′E Long; 27°35.72′N Lat.)*	1600	0.470 ± 0.045	N.D.	0.046 ± 0.001	1.835 ± 0.014	0.192 ± 0.007	1.835 ± 0.023	0.364 ± 0.007	0.369 ± 0.001
Lalitpur 2 (85°22.70′E Long.; 27°35.72′N Lat)	1600	0.520 ± 0.032	N.D.	0.047 ± 0.002	2.431 ± 0.032	0.208 ± 0.006	2.431 ± 0.054	0.382 ± 0.004	0.385 ± 0.009
Gorkha 1(84°38.74′E Long; 28°01.39′N Lat.)*	600	8.136 ± 0.049	N.D.	0.092 ± 0.003	0.363 ± 0.012	0.338 ± 0.006	1.246 ± 0.021	0.389 ± 0.015	0.381 ± 0.011
Gorkha 2 (84°3.74′E Long; 27°92.28′N Lat)	600	3.891 ± 0.041	N.D.	0.093 ± 0.004	0.277 ± 0.014	0.295 ± 0.008	1.695 ± 0.016	0.383 ± 0.008	0.384 ± 0.010
Gorkha 3 (84°3.74′E Long; 28°02′NLat)	600	2.710 ± 0.042	N.D.	0.063 ± 0.002	0.186 ± 0.009	0.221 ± 0.011	0.597 ± 0.012	0.382 ± 0.006	0.387 ± 0.013
Lamjung (84°2.66′E Long; 28°14.57′N Lat)*	720	0.236 ± 0.035	0.030 ± 0.001	N.D.	N.D.	N.D.	0.295 ± 0.009	0.321 ± 0.009	0.022 ± 0.000
Kaski-1 (83°9.33E′ Long; 28°16.99′NLat)*	670	2.820 ± 0.048	0.290 ± 0.009	0.054 ± 0.001	0.271 ± 0.008	0.112 ± 0.004	0.981 ± 0.010	0.384 ± 0.000	0.384 ± 0.011
Kaski-2 (83°9′E Long; 28°18′N Lat)*	850	1.660 ± 0.046	N.D.	0.054 ± 0.002	0.124 ± 0.009	0.113 ± 0.008	0.772 ± 0.023	0.383 ± 0.005	0.381 ± 0.013
Kaski-3 (83°9.20′E Long; 28°15′NLat)	850	1.535 ± 0.040	0.275 ± 0.014	0.063 ± 0.002	0.062 ± 0.002	0.041 ± 0.001	0.484 ± 0.009	0.389 ± 0.012	0.382 ± 0.011
Kaski-4 (83°9.20′E Long; 28°15′N Lat.)	850	2.471 ± 0.025	0.279 ± 0.018	0.049 ± 0.001	0.056 ± 0.001	0.042 ± 0.001	0.469 ± 0.009	0.331 ± 0.008	0.334 ± 0.013
Kaski-5 (83°51.13′ELong; 28°18.012′NLat.)*	1800	2.051 ± 0.046	0.275 ± 0.015	0.056 ± 0.003	0.377 ± 0.021	0.190 ± 0.002	2.752 ± 0.042	0.388 ± 0.014	0.388 ± 0.008
Kaski-6 (83°51.13′E Long; 28°18.102′N Lat.)	1800	1.503 ± 0.035	N.D.	0.052 ± 0.002	0.194 ± 0.007	0.120 ± 0.003	1.594 ± 0.021	0.357 ± 0.021	0.357 ± 0.009
Kaski-7 (83°51.13′E Long; 28°18.102′N Lat.)	1800	0.628 ± 0.043	0.663 ± 0.022	N.D.	N.D.	N.D.	0.241 ± 0.006	0.361 ± 0.010	0.360 ± 0.009
Kaski-8 (83°52.15′E Long.; 28°20.08′N Lat.)*	2000	0.331 ± 0.023	0.481 ± 0.016	N.D.	N.D.	N.D.	0.171 ± 0.009	N.D.	N.D.
Mean		1.884	0.109	0.050	0.380	0.145	1.200	0.357	0.333

N.D. = amount was under LOQ; '*' germplasm used for cultivation.

Table 4
Amount of selected phytoconstituents in Western Nepal wild plants, expressed as % ±SD.

Site (Location)	Altitude (m)	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O-glucuronide	Kaempferol	Quercetin
Pyuthan-1 (82°7.99′E Long; 28°2.28′N Lat)*	1300	0.606 ± 0.013	N.D.	0.046 ± 0.002	0.353 ± 0.012	0.220 ± 0.010	0.851 ± 0.024	0.372 ± 0.009	0.371 ± 0.011
Pyuthan-2 (82°7.99′E Long; 28°2.28′N Lat.)	1300	0.591 ± 0.012	N.D.	0.044 ± 0.001	0.702 ± 0.017	0.272 ± 0.008	0.309 ± 0.009	0.371 ± 0.011	0.372 ± 0.014
Dang−1 (82°4.13′E Long; 27°9.5′N Lat)	650	0.997 ± 0.023	N.D.	0.053 ± 0.000	0.161 ± 0.011	0.051 ± 0.002	0.603 ± 0.019	0.366 ± 0.014	0.366 ± 0.006
Dang-2 (82°4.13′E Long; 27°9.5′N Lat.)*	650	2.244 ± 0.032	N.D.	0.068 ± 0.002	0.179 ± 0.009	0.076 ± 0.003	0.435 ± 0.011	0.410 ± 0.009	0.410 ± 0.021
Dang-3 (82°4.13′E Long; 27°9.5′N Lat)	650	1.195 ± 0.041	N.D.	0.052 ± 0.001	0.180 ± 0.007	0.073 ± 0.002	0.639 ± 0.022	0.387 ± 0.008	0.387 ± 0.010
Surkhet-1 (81°5.99′E Long.; 28°6.25′N Lat.)	675	N.D.	N.D.	0.057 ± 0.002	0.115 ± 0.004	N.D.	0.336 ± 0.008	N.D.	N.D.
Surkhet-2 (81°5.99′E Long.; 28°6.25′N Lat.)*	675	N.D.	N.D.	0.043 ± 0.001	0.067 ± 0.002	N.D.	0.090 ± 0.004	N.D.	N.D.
Banke (81°3.99′E Long; 28°0.73′N Lat.)*	150	2.993 ± 0.013	N.D.	0.062 ± 0.003	0.253 ± 0.009	N.D.	0.678 ± 0.010	0.371 ± 0.009	0.371 ± 0.008
Kanchanpur (80°2.25′E Long; 29°0.50′Lat.)*	120	0.792 ± 0.012	0.404 ± 0.009	0.043 ± 0.000	N.D.	N.D.	0.041 ± 0.001	N.D.	0.380 ± 0.007
Mean		1.046	0.045	0.052	0.227	0.091	0.442	0.296	0.295

N.D. = amount was under LOQ; '*' germplasm used for cultivation.

was: 0–8.5 min, linear gradient from 12 to 26% B; 8.5–11 min, isocratic conditions at 26% B; 11–16 min, linear gradient from 26 to 40% B; 16–45 min, linear gradient from 40 to 50% B; 45–50 min, linear gradient from 50 to 100% B. Flow rate was 1 mL/min and injection volume 20 μL. The ELSD detector temperature was 50 °C, nitrogen pressure 2.2 bars, and the gain level 10 (a.u.).

Calibration curves were obtained by preparing standard solutions, as listed in Table 1. Asiaticoside and asiatic acids were determined with the ELSD detector. Chlorogenic, chicoric and rosmarinic acids were determined with the DAD at 330 nm, and at 350 nm for kaempferol, quercetin and quercetin 3-O-glucuronide. HPLC chromatograms of standard compounds and a selected sample are shown in Figs. 1 and 2, respectively.

2.8. Regression curves, limits of detection and quantification

Regression curves were performed at five concentration levels by the external method, each concentration being injected in triplicate. Calibration curves were constructed by plotting peak area (X) vs concentration of standard solutions (Y, μg/mL). A good correlation ($R^2 > 0.9976$) was achieved over relatively wide concentration ranges for all analytes. The limit of detection (LOD) was calculated by analysing standard solutions of decreasing concentrations, in order to establish the lowest concentration that could be detected with a signal-to-noise (S/N) ratio of 3. The limit of quantification (LOQ) was defined as the lowest concentration that the method could quantify (S/N = 10). Data are presented in Table 1.

3. Results and discussion

3.1. Determination of terpene and phenol constituents in wild plants

In order to evaluate the contents of bioactive components, seven compounds were selected as markers. Asiatic acid and asiaticoside were examined for terpene, and quercetin, quercetin 3-O-glucuronide, kaempferol, and rosmarinic, chicoric and chlorogenic acids for phenol constituents. HPLC analysis revealed considerable variability in the contents of these bioactive components (Tables 2–4). Considering their mean contents in samples from the three regions, those from central Nepal contained the highest amounts of all metabolites (Table 2). Asiaticoside from Central Nepal samples ranged from 0.24 to 8.13%, with a mean value of 1.88% (d.w). In the Eastern and Western Nepal samples, mean values were 1.20% and 1.05% respectively The mean asiaticoside content of Gorkha samples was 4.92% (range 2.72–8.14% d.w.), similar to that reported for *C. asiatica* from Madagascar (Randriamampionona et al., 2007), in which the amount of asiaticoside ranged from 2.67 to 6.42% (d.w). Comparative study within 10 ecotypes from various regions in India showed a correlation between genomic diversity and asiaticoside contents (Das and Mallick, 1991) and the highest amount of asiaticoside was 0.11% d.w.. Gupta et al. (1999) also reported variable asiaticoside contents in five lines of *C. asiatica* collected from a field trial in India, mean levels varying from 0.42 to 1.17. The amount of asiatic acid in all the wild collected plants was very low. Small amounts of phenol constituents were detected in all samples, with small differences according to geographic origin. Central Nepal samples contained higher levels than the others (Table 3). Significant differences were observed in chlorogenic acid and quercetin 3-O-glucuronide contents

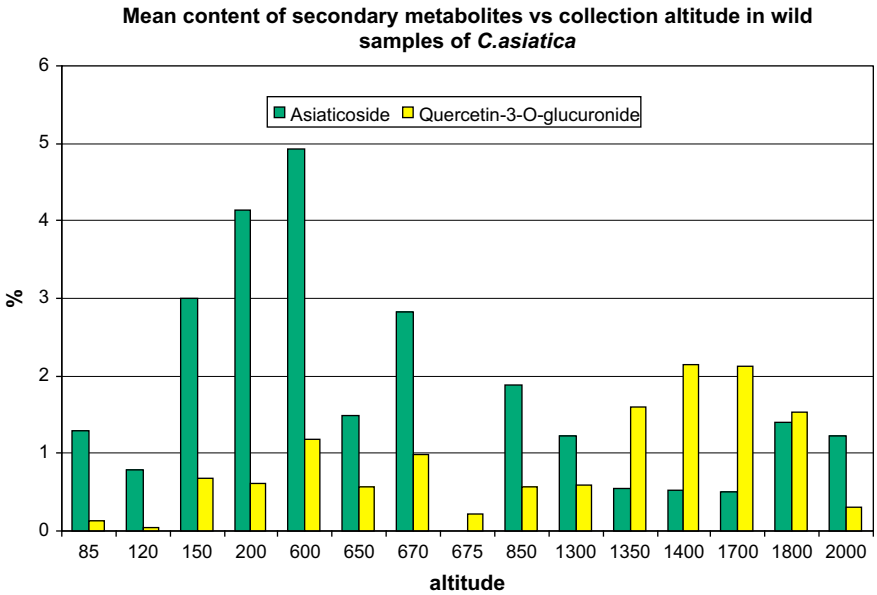

Fig. 3. Correlation between altitude of collection and asiaticoside and quercetin-3-O-glucuronide content in wild samples of *C. asiatica*.

Table 5
Amount of selected phytoconstituents in cultivated plants, expressed as % ±SD. Altitude of cultivation site, 1350 m a.s.l.

Origin of germplasm	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O-glucuronide	Kaempferol	Quercetin
Ilam −1 (87°90.32′E Long.; 26°72.80′N Lat.)	0.908 ± 0.012	0.923 ± 0.012	N.D.	0.065 ± 0.000	N.D.	0.194 ± 0.008	N.D.	N.D.
Ilam-2 (87°90.32′E Long.; 26°72.80′N Lat)	0.159 ± 0.004	0.347 ± 0.007	N.D.	N.D.	N.D.	0.246 ± 0.007	N.D.	N.D.
Sunsari-1 (87°1.61′E Long.; 26°41.28′N Lat)	0.541 ± 0.023	0.276 ± 0.010	0.006 ± 0.000	0.044 ± 0.001	N.D.	0.265 ± 0.005	N.D.	N.D.
Sunsari-2 (87°1.61′E Long.; 26°41.28′N Lat)	1.259 ± 0.034	1.137 ± 0.029	N.D.	0.045 ± 0.002	N.D.	0.080 ± 0.002	N.D.	N.D.
Dhankuta-1 (87°3.54′E Long.; 26°77.70′N Lat)	0.992 ± 0.016	0.759 ± 0.009	N.D.	0.044 ± 0.002	N.D.	0.251 ± 0.09	N.D.	N.D.
Dhankuta-2 (87°3.54′E Long.; 26°77.70′N Lat)	0.472 ± 0.012	0.132 ± 0.008	N.D.	N.D.	N.D.	0.284 ± 0.009	N.D.	N.D.
Jhapa-1 (88°3.05′E Long.; 26°31.6′N Lat.)	0.976 ± 0.021	0.333 ± 0.011	N.D.	N.D.	N.D.	0.167 ± 0.010	N.D.	N.D.
Jhapa-2 (88°3.05′E Long.; 26°31.6′N Lat.)	0.197 ± 0.008	0.366 ± 0.09	N.D.	N.D.	N.D.	0.227 ± 0.005	N.D.	N.D.
Kathmandu −1 (85°17.32′E Long.; 27°40.20′N Lat.)	0.198 ± 0.011	0.454 ± 0.021	N.D.	0.057 ± 0.000	N.D.	0.253 ± 0.008	N.D.	N.D.
Kathmandu-2 (85°17.32′E Long.; 27°40.20′N Lat.)	0.149 ± 0.009	N.D.	N.D.	0.065 ± 0.002	N.D.	0.191 ± 0.0012	N.D.	N.D.
Makwanpur 1 (85°0.32′E Long.; 27°37.5′NLat)	1.728 ± 0.025	0.500 ± 0.011	N.D.	N.D.	N.D.	0.348 ± 0.011	N.D.	N.D.
Makwanpur 2 (85°0.32′E Long; 27°37.5′N Lat)	1.093 ± 0.032	0.735 ± 0.016	N.D.	N.D.	N.D.	0.278 ± 0.009	N.D.	N.D.
Makwanpur 1(85° 10.05′E Long., 27°29.14′ Lat.)	0.109 ± 0.007	1.092 ± 0.021	N.D.	0.064 ± 0.002	N.D.	0.275 ± 0.007	N.D.	N.D.
Makwanpur 2 (85° 10.05′E Long., 27°29.14′ Lat.)	0.135 ± 0.005	1.001 ± 0.017	N.D.	N.D.	N.D.	0.076 ± 0.002	N.D.	N.D.
Parbat-1 (83°6.67′E Long, 28° 18.9′ Lat.)	1.399 ± 0.031	0.761 ± 0.016	N.D.	N.D.	N.D.	0.361 ± 0.007	N.D.	N.D.
Parbat-2 (83°6.67′E Long, 28° 18.9′N Lat.)	0.157 ± 0.008	1.107 ± 0.009	N.D.	N.D.	N.D.	0.172 ± 0.008	N.D.	N.D.
Kaski 1 (83°51.13′E Long.; 28°18.012′N Lat.)	0.415 ± 0.001	N.D.	N.D.	0.059 ± 0.002	N.D.	0.513 ± 0.015	N.D.	N.D.
Kaski 2 (83°51.13′E Long.; 28°18.012′N Lat.)	0.091 ± 0.002	0.038 ± 0.001	N.D.	N.D.	N.D.	0.225 ± 0.009	N.D.	N.D.
Lalitpur-1 (85°22.70′E Long.; 27°35.72′N Lat.)	0.246 ± 0.008	0.645 ± 0.008	0.041 ± 0.003	0.091 ± 0.003	N.D.	0.495 ± 0.012	N.D.	N.D.
Lalitpur −2 (85°22.70′E Long.; 27°35.72′N Lat)	0.101 ± 0.005	0.736 ± 0.014	N.D.	0.041 ± 0.001	N.D.	0.484 ± 0.017	N.D.	N.D.
Kaski (2000) 1 (83°52.15′E Long.; 28°20.08′N Lat.)	1.043 ± 0.012	0.095 ± 0.003	N.D.	0.123 ± 0.007	N.D.	0.291 ± 0.0010	N.D.	N.D.
Kaski (2000).-2 (83°52.15′E Long.; 28°20.08′N Lat.)	0.691 ± 0.004	0.228 ± 0.011	N.D.	0.071 ± 0.003	N.D.	0.232 ± 0.011	N.D.	0.057 ± 0.002
Rasuwa-1 (85° 2.9′E Long.; 28° 05′N Lat.)	0.127 ± 0.005	0.985 ± 0.010	N.D.	0.050 ± 0.002	N.D.	0.261 ± 0.005	N.D.	N.D.
Rasuwa-2 (85° 2.9′E Long.; 28° 05′N Lat)	0.118 ± 0.003	0.680 ± 0.009	N.D.	0.056 ± 0.003	N.D.	0.136 ± 0.007	N.D.	N.D.
Lamjung 1 (84°2.66′E Long; 28°14.57′N Lat)	0.433 ± 0.0012	N.D.	N.D.	0.094 ± 0.004	N.D.	0.308 ± 0.010	N.D.	N.D.
Lamjung 2 (84°2.66′E Long.; 28°14.57′N Lat)	0.893 ± 0.018	0.214 ± 0.008	N.D.	0.050 ± 0.002	N.D.	0.237 ± 0.008	N.D.	N.D.
Gorkha 1 (84°38.74′E Long; 28°01.39′′N Lat)	1.911 ± 0.032	1.107 ± 0.022	N.D.	0.055 ± 0.002	N.D.	0.277 ± 0.007	N.D.	N.D.
Gorkha 2 (84°38.74′E Long.; 28°01.39′N Lat)	1.642 ± 0.027	0.117 ± 0.003	N.D.	N.D.	N.D.	0.494 ± 0.012	N.D.	N.D.
Pyuthan-1 (82°7.99′E Long.; 28°2.28′N Lat)	0.693 ± 0.017	0.989 ± 0.012	N.D.	N.D.	N.D.	0.056 ± 0.002	N.D.	0.401 ± 0.009
Pyuthan-2 (82°7.99′E Long; 28°2.28′N Lat)	0.157 ± 0.009	0.321 ± 0.011	N.D.	N.D.	N.D.	N.D.	N.D.	0.281 ± 0.010
Dang-1 (82°4.13′E Long.; 27°9.5′N Lat.)	1.349 ± 0.016	0.978 ± 0.010	N.D.	0.067 ± 0.003	N.D.	N.D.	N.D.	0.263 ± 0.011
Dang-2 (82°4.13′E Long.; 27°9.5′N Lat.)	1.039 ± 0.019	0.831 ± 0.021	N.D.	0.063 ± 0.002	N.D.	N.D.	N.D.	0.120 ± 0.007
Surkhet-1 (81°5.99′E Long.; 28°6.25′N Lat.)	1.349 ± 0.000	0.250 ± 0.009	N.D.	N.D.	N.D.	N.D.	N.D.	0.222 ± 0.008
Surkhet-2 (81°5.99′E Long.; 28°6.25′N Lat.)	0.189 ± 0.007	0.897 ± 0.023	N.D.	N.D.	N.D.	N.D.	N.D.	0.227 ± 0.012
Banke-1 (81°3.99′E Long; 28°0.73′N Lat.)	0.436 ± 0.010	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.246 ± 0.013
Banke-2 (81°3.99′E Long.; 28°0.73′N Lat.)	1.319 ± 0.024	0.710 ± 0.007	N.D.	0.057 ± 0.002	N.D.	N.D.	N.D.	0.233 ± 0.006
Kanchanpur 1 (80°2.25′E Long.; 29°0.50′ N Lat.)	1.151 ± 0.021	0.588 ± 0.008	N.D.	0.044 ± 0.002	N.D.	N.D.	N.D.	0.229 ± 0.009
Kanchanpur 2 (80°2.25′E Long; 29°0.50′N Lat.)	0.566 ± 0.012	0.508 ± 0.006	N.D.	N.D.	N.D.	N.D.	N.D.	0.210 ± 0.008
Kailali-1 (80° 2.25′E Long; 28°7.31′N Lat)	0.120 ± 0.005	0.007 ± 0.000	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Kailali 2 (80° 2.25′E Long; 28°7.31′N Lat)	0.481 ± 0.008	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.084 ± 0.004
Chitwan 1 (84°3.22′E Long.; 27°60.71′N Lat)	1.186 ± 0.021	0.720 ± 0.017	N.D.	0.059 ± 0.002	N.D.	0.241 ± 0.008	N.D.	0.104 ± 0.005
Chitwan 2 (84°3.22′E Long.; 27°60.71′N Lat.)	1.169 ± 0.016	0.310 ± 0.007	N.D.	0.062 ± 0.002	N.D.	0.203 ± 0.007	N.D.	0.088 ± 0.003
Mean	0.700	0.521	0.005	0.042	0.015	0.200	0.014	0.081

Table 6
Amount of selected phytoconstituents in cultivated plants in differing environmental conditions, expressed as % ±SD.

Treatment	Asiaticoside	Asiatic acid	Chicoric acid	Chlorogenic acid	Rosmarinic acid	Quercetin 3-O-glucuronide	Kaempferol	Quercetin
Watering daily	0.120 ± 0.007	N.D.	N.D.	N.D.	N.D.	0.274 ± 0.009	N.D.	N.D.
Watering once in a week	0.165 ± 0.008	0.934 ± 0.014	N.D.	N.D.	N.D.	0.070 ± 0.003	N.D.	N.D.
Daily watering	0.213 ± 0.010	N.D.	N.D.	N.D.	N.D.	0.314 ± 0.010	N.D.	N.D.
Watering at three-day intervals	0.193 ± 0.007	N.D.	N.D.	0.072 ± 0.002	N.D.	0.273 ± 0.007	N.D.	N.D.
Greatly disturbed	0.151 ± 0.011	0.955 ± 0.018	N.D.	0.057 ± 0.002	N.D.	0.096 ± 0.003	N.D.	N.D.
Moderately disturbed	0.159 ± 0.004	0.269 ± 0.008	0.024 ± 0.001	N.D.	N.D.	0.252 ± 0.008	N.D.	N.D.
Undisturbed	0.158 ± 0.005	0.239 ± 0.007	0.006 ± 0.000	N.D.	N.D.	0.329 ± 0.011	N.D.	N.D.
Grown in shade	0.158 ± 0.009	1.272 ± 0.031	N.D.	N.D.	N.D.	0.167 ± 0.009	N.D.	N.D.
Grown in partial shade	0.179 ± 0.008	N.D.	N.D.	0.052 ± 0.001	N.D.	0.103 ± 0.005	N.D.	N.D.
Grown in light	0.256 ± 0.011	0.043 ± 0.002	N.D.	0.069 ± 0.002	N.D.	0.022 ± 0.001	N.D.	N.D.
0% sand	0.217 ± 0.012	0.494 ± 0.006	N.D.	0.066 ± 0.002	N.D.	0.104 ± 0.003	N.D.	N.D.
20% sand	0.271 ± 0.006	0.906 ± 0.012	N.D.	N.D.	N.D.	0.092 ± 0.004	N.D.	N.D.
4 sand	0.102 ± 0.004	0.671 ± 0.013	N.D.	N.D.	N.D.	0.123 ± 0.007	N.D.	N.D.
60% sand	0.122 ± 0.006	0.075 ± 0.002	N.D.	N.D.	N.D.	0.148 ± 0.005	N.D.	N.D.
80% sand	0.134 ± 0.005	N.D.	N.D.	N.D.	N.D.	0.205 ± 0.005	N.D.	N.D.
100% sand	0.585 ± 0.016	0.724 ± 0.024	N.D.	0.038 ± 0.001	N.D.	0.274 ± 0.010	N.D.	N.D.
10 g compost/pot	0.096 ± 0.003	N.D.	N.D.	0.031 ± 0.002	N.D.	0.269 ± 0.007	N.D.	N.D.
20 g compost/pot	0.125 ± 0.004	0.092 ± 0.003	N.D.	0.108 ± 0.007	N.D.	0.150 ± 0.006	N.D.	N.D.
Urea	0.223 ± 0.009	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Control	0.097 ± 0.002	0.054 ± 0.002	N.D.	0.086 ± 0.003	N.D.	0.230 ± 0.009	N.D.	N.D.

N.D. = amount was under LOQ.

among samples. In Central and Western Nepal samples, chlorogenic acid content was four times higher than in Eastern Nepal ones. Quercetin 3-O-glucuronide was higher in Central Nepal samples (mean value 1.20% d.w.).

Correlations were made, considering sampling site altitudes and amounts of asiaticoside and quercetin 3-O-glucuronide. Samples growing at 200–600 m a.s.l. had higher asiaticoside contents. Collection altitude and asiaticoside content were inversely related with higher triterpene contents in samples collected at 150–600 m. Thus, a negative correlation was observed between altitude and asiaticoside contents, whereas the contrary occurred for quercetin-3-O-glucuronide (Fig. 3). The amounts of flavonoids had previously been related to altitude by other authors (Ganzera et al., 2008; Rieger et al., 2008).

The first aim of this work was achieved, and these results helped us in selecting high-terpene producing plants. Seeds from the plants grown in the Gorkha and Chitwan districts were/will be considered as starting materials for *C. asiatica* cultivation in Nepal.

3.2. Determination of terpene and phenol constituents in cultivated plants

Some germplasm used to analyse wild samples was also applied to cultivated experimental plots in Kathmandu. The amounts of secondary metabolites were far lower than those of wild plants (see Table 5). Among cultivated samples, germplasm collected from the Gorkha district had the highest asiaticoside contents (1.9%). Samples from Sunsari, Makwanpur, Chitwan, Parbat, Kaski, Dang Surkhet and Mahendranagar also had asiaticoside higher than 1% (Table 5). Asiatic acid varied greatly, ranging from non-detectable (less than 0.05%) to 1.14%, with a mean value of 0.52%; it was relatively higher in cultivated than in wild samples. Significant differences were also observed in quercetin 3-O-glucuronide in cultivated samples (Table 5). Other bioactive components, such as chicoric and chlorogenic acids, kaempferol and quercetin were detected in small amounts in all samples, both experimentally grown and wild.

3.3. Determination of terpene and phenol constituents in cultivated plants in differing environmental conditions

Some experimental pots were also prepared with Kathmandu (Kirtipur) germplasm, in order to study the influence of differing environmental conditions on the active constituents of *C. asiatica* (Table 6). Variations in watering and level of disturbance did not significantly affect the amounts of metabolites. Light exposure affected the amount of terpenes: 70% shade-grown plants had the lowest asiaticoside and highest asiatic acid contnts. Further trials involved cultivating plants in soils containing various fertilisers. This procedure was critical, since plants cultivated in compost-added soil had lower secondary metabolites. Samples grown in sand alone contained the same amount of asiaticoside as the original wild type germplasm, whereas all the other cultivated samples had lower amounts. Metabolites were not greatly affected by different levels of disturbance. Plants also produced more terpenes when grown in sand-rich soils.

These results may be considered as preliminary indications for the cultivation of *C. asiatica*.

4. Conclusions

Our findings showed great variations in secondary metabolite contents in analysed samples, suggesting future study such as evaluation of genetic variations among different plant populations. The present study reveals the high levels of metabolites and better performance of *C. asiatica* collected in Central Nepal than in Eastern and Western regions. According to these findings, it is emphasised that *C. asiatica* from Central Nepal is the best choice for collection and further cultivation. Cultivated *C. asiatica* could represent a future source of this medicinal plant, helping the Nepalese market by producing high-quality vegetal material and reducing the need for spontaneous collections, thus helping to preserve natural biodiversity and protecting the ecosystem from uncontrolled collection of wild populations of plants.

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RESEARCH ARTICLE

Seed Germination responses of the medicinal herb *Centella asiatica*

Anjana Devkota* and Pramod Kumar Jha

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

* Corresponding author: devkotaa@gmail.com; pkjhaprof@gmail.com; phone: 977-1-4331322, fax: 977-1- 4333515
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ABSTRACT

The effect of several environmental factors on germination of medicinal herb *Centella asiatica* was investigated. Freshly harvested seeds of *C. asiatica* did not germinate even after gibberellic acid (GA_3) treatment and exposure to different treatments with light qualities, while two-three months old seeds exhibited germination (82%) without pre-treatment at warm environment (25 -30°C). GA_3 treatment induced germination by two weeks earlier than in control. Germination was significantly ($p=0.001$) higher in red and white light than in blue and far red light. In addition, germination of *C. asiatica* was sensitive towards the salt stress and was significantly inhibited at 6500 ppm NaCl. The leaf leachates from invasive weeds *Chromolaena odorata*, *Ageratum conyzoides*, *Parthenium hysterophorus* and *Xanthium strumarium* showed inhibitory effects on seed germination of *C. asiatica. Parthenium hysterophorus* had significant effect ($p<0.001$) on seed germination. These data contribute for the establishing of an efficient protocol for *C. asiatica* cultivation.

Key Words: 2,3,5-triphenyltetrazolium chloride, Salinity, Leaf leachate, weeds, gibberellic acid

INTRODUCTION

Centella asiatica (L.) Urban (Fam. Apiaceae) is an important medicinal plant in Nepal (Devkota and Jha, 2008). Extract of *Centella* exerted anti-inflammatory and antifilarial effects and is used as memory enhancer and wound healer (Shukla et al., 1999). Clinical trials have also shown that it can help those with chronic venous insufficiency (Brinkaus et al., 2000). Medicinal properties of the plant led to its over exploitation. *Centella asiatica* propagates vegetatively as well as by seeds. Seeds offer genetic variation and ease of portability and storage as compared to vegetative propagules apart of resilience to draught (Brock et al., 2003).

Uniform stand establishment for *Centella* production is essential for maintaining profitable yields. Poor stands result from several factors including seed quality, environment, water, soil quality, site preparation, plant uniformity, irrigation practices and other management practices. Excessive use of chemical fertilizers and pesticides contribute to an increase of soil salinity which is one of the major obstacles in increasing crop production in the world (Serrano et al., 1999). It is important to establish seed germination requirements of important species such as *C. asiatica*, in order to understand the possible role of different environmental factors for the establishment in nature. Therefore this study focused on seed attributes and effects of salinity, pretreatments (i.e. warm water, 10%

HNO_3, GA_3 treatments), extracts of alien species and light quality on seed germination of *C. asiatica*.

MATERIALS AND METHODS

Seed source and physical attribute: The *Centella* seeds were collected from natural population (agricultural land in Kirtipur during fallow period). Climate of Kirtipur, Kathmandu is sub tropical and monsoonic and the soil has slightly acidic nature. The seeds were collected during June 2006 and 2007. Seeds were dried in shade for a week and stored in hermetically sealed polythene pouches (Copeland, 1976) under ordinary storage condition at room temperature for subsequent germination studies.

Seed output per unit area was measured according to Zobel et al., (1987). The seed size was determined by measuring scale, whereas seed mass was determined by using an electric balance (0.001g).

Seed viability: The seed coverings were removed manually and the decoated seeds were soaked in distilled water and kept in dark for 24h before being treated with 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) for assessing the effects of various durations of storage on viability (Misra,1968).

Germination test: Germination of *Centella asiatica* was evaluated using 2-3 months old seeds by placing seeds in a Petri dish containing two layers of Whatman No. 1 filter paper, moistened with 10 ml of distilled water or a treatment solution. The Petri dishes were placed on a table in the laboratory at room temperature. The average daily maximum and minimum room temperatures during the experiment were 30 and 20 ^oC, respectively. The Petri dishes were wrapped in two layers of aluminium foil to determine germination in complete darkness. All seeds except for pre-treatment experiment were soaked in 10 ppm GA_3 for 30 min before incubating in Petri dish for enhancing germination. Seed germination was monitored 2 days after the beginning of the experiment, with the criterion for germination being visible protrusion of the radicle. All sets of treatment and control contained four replicates, each of 30 seeds.

Effects of pre-treatments: Presoaking treatments used in the study were: different concentration of GA_3, warm water, cold water and 10% HNO_3. Before placing in Petri dishes, seeds were soaked in 10,20,30,40, 50 and 100 ppm GA_3 solution; 40 and 60^oC warm water and cold water (at room temperature) for 30 min. For 10% HNO_3 treatment, after soaking the seeds were washed with distilled water three times before incubation in Petri dishes.

Effects of light: To study the effects of coloured light; petri dishes with seeds were covered by red and blue cellophane plastic till the completion of experiment.

Effect of Salt Stress: In order to determine the influence of salt stress on germination seeds of C. *asiatica* were incubated in sodium chloride (NaCl) solutions of 0,500, 1500, 2500, 3500, 4500 and 6500 ppm . The non-germinated seeds at 6500 ppm were transferred to Petri dishes containing 5 ml of distilled water and placed in the incubator as described previously. The seeds were rinsed before being transferred.

Phytotoxic effects of leaf leachates of alien plants: Four alien species, *Chromolaena odorata, Parthenium hysterophorus, Ageratum conyzoides* and *Xanthium strumarium*, were collected randomly in vegetative stage in a sunny mid day of July 2007 from Kirtipur. Leachates were prepared by immersing 10 g each of fresh plant materials (leaf, stem) in 100 ml of distilled water in beaker separately and kept for 24 h at room temperature and filtered using muslin cloth as recommended by Tukey and Mecklenburg (1964). This filtrate was taken as stock solution with 10% of concentration and different concentration solution (i.e. 1 and 5%) were made by dilution with distilled water and used for Petri dish bioassay with *C. asiatica* seeds.

Statistical Analyses: All experiments were conducted in a randomized complete block design. Treatments of each experiment were replicated three times and all experiments were repeated. The data from the repeated experiments were pooled before being subjected to ANOVA.

RESULTS

Seed attributes and viability: As shown in Table 1 *Centella asiatica* had 15 seeds per ramet which are brownish in colour

and slightly kidney-shaped. The average length and breadth of seeds were 2.84 and 1.81 mm, respectively. The mass of 100 air dried and water imbibed (24h) seeds were also determined. TTC test indicated 82% viability in freshly harvested seeds of *C. asiatica*. Seeds viability deteriorated as duration of storage increased and they became non-viable after storage for thirty months (Figure 1). Freshly harvested seeds of *C. asiatica* did not germinate even after GA_3 treatment and incubation under different light qualities, whereas two-three months old seeds exhibited germination (82%) without pre-treatment at warm environment (25-30°C).

Table 1. *Centella asiatica* seeds output and characteristics

Seed attributes [a]	Values
Seed colour	Brownish
Seed shape	Slightly kidney-shaped
Seed size	
i..seed width(mm)	i.1.81±0.2 *
ii. seed length(mm)	ii. 2.84±0.25 *
No. of seeds/fruit	3.0±1.0 *
No of fruits/ramet	5.0±2.0 *
Seed output/ramet	15.0±2.0 *
Plant density/m^2	32.0±3.0 **
Seed production/m^2	480.0±8.0 **
Weight of 100 air dried seeds (g)	0.13±0.02
Weight of 100 water imbibed seeds (g)	0.38±0.08

The data represent mean ± SE (* n=100; ** n=30)

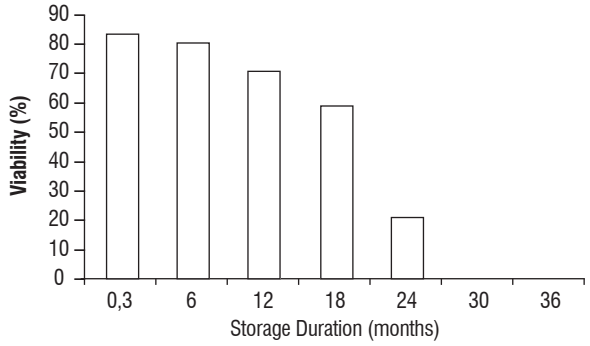

Figure 1.Viability of *C. asiatica* seeds on different storage duration.

Effects of different factors on germination: The effects of ten different pre-treatments on germination of seeds of *Centella asiatica* are shown in Figure 2. The germination percentage varied significantly among the treatments. Germination of seeds pre-treated with 60ºC water were significantly lower than germination of seeds pre-treated with 10% HNO_3 and seeds treated with different concentration of GA_3. Among the different treatments of GA_3, soaking seeds in 10 ppm resulted in highest germination and was followed by 20 ppm. Increasing the concentration had no effect on germination (Figure 2). However GA_3 treatment induced germination by nearly a week earlier than other treatments (Table 2) where seed germination occurred after second week of incubation. Treatments with 10% HNO_3 and warm water treatments (40 and 60º C) promoted the seed germination from third week of incubation, while it was started from fourth week in control (distilled water; Table 2).

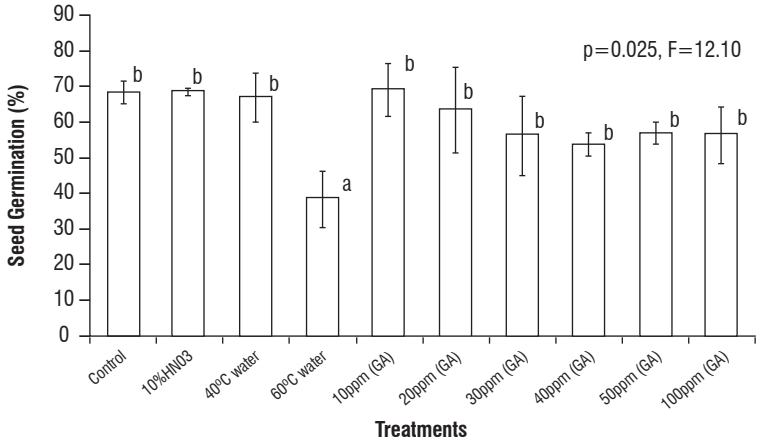

Figure 2. Effects of different pre-treatments on seed germination of *C. asiatica* after six weeks (bars marked with the same letter are not significantly different from the control, p>0.05).

Table 2. Effects of different pre-treatments on germination of *Centella asiatica* seeds.

Treatments	Seed Germination (%)					
	week					
	1st	2nd	3rd	4th	5th	6th
Control	0	0	0	4	48	68
10% HNO3	0	0	20	65	65	68.33
40ºC water	0	0	8	40	56	66.66
60ºC water	0	0	8	18	30	38.33
10 ppm GA_3	0	20	65	65	65	69
20 ppm GA_3	0	5	35	35	45	63.33
30 ppm GA_3	0	5	35	35	45	56
40 ppm GA_3	0	20	40	50	52	53.33
50 ppm GA_3	0	15	50	50	56	56.66
100 ppm GA_3	0	10	20	40	40	56

Effect of coloured light on germination: Significant differences ($p<0.001$) in germination were observed when seeds were exposed to the different wave lengths of white light (blue, red and far red light) (Figure 3). The germination was slightly higher in red light and inhibited in blue and far red lights. The seeds were defective on germination in dark conditions.

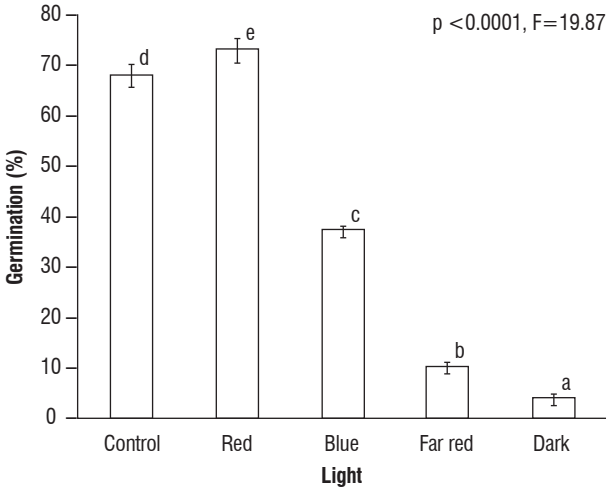

Figure 3. Final germination percentages of *Centella asiatica* seeds in different coloured light (observation at sixth week).

Effect of Salt Stress on Germination: Salinity significantly affected the germination percentage of *C. asiatica* ($p<0.001$) (Figure 4). At salinities up to 3500 ppm NaCl germination rate ranged 70-73 %. The germination was inhibited by 57 and 89% at 4500ppm and 5500ppm NaCl respectively, and completely abolished at 6500 ppm salinity. When non-germinated seeds were removed from 6500 ppm and placed in distilled water there was no germination recorded, indicating that seeds were irreversibly severely affected by exposure to conditions of high salinity and were not able to germinate later on even in favourable conditions.

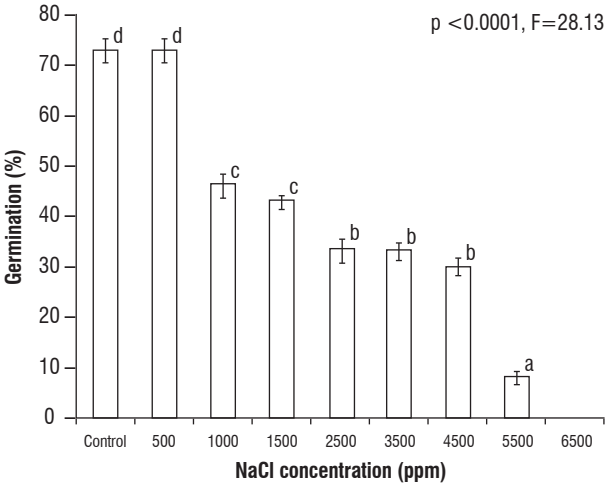

Figure 4. Germination of seeds of *Centella asiatica* in different concentration of NaCl (bars marked with the same letter are not significantly different at $p>0.05$).

Phytotoxic effects of leaf leachates of alien plants: The germination of *Centella asiatica* seeds was sensitive to leaf leachates of different alien invasive weeds (Table 3) when

compared to control. Highest percentage of germination of seeds was observed at control (88%). Out of the four plants studied, concentrated leachates of *Parthenium hysterophorus* leachates exhibited drastic significantly higher ($p<0.001$) inhibitory effects on germination (only 20% germination), and followed by *Ageratum conyzoides* 10% extract where only 40% seeds germinated, while lowest inhibitory effect on germination was observed using *Chromolaena odorata* (Table 3).

Table 3. Effects of leaf leachates of some weeds plants on seed germination of *C. asiatica.*

Treatments	Germination (%) ± S.D.					
	1st week	2nd week	3rd week	4th week	5th week	6th week
Control (0%)	0	0	5±1.0	61.0±1.0	88±1.0	88±1.0
Chromolaena odorata (1%)	0	0	1.0±1.0	35.0±2.0	75±2.0	75±2.0
Chromolaena odorata (5%)	0	0	0	20±1.0	50±1.0	50**±1.0
Chromolaena odorata (10%)	0	0	0	26±1.0	56±1.0	56**±2.0
Parthenium hysterophorus (1%)	0	0	0	30±2.0	63±2.0	63*±2.0
Parthenium hysterophorus (5%)	0	0	0	18±1.0	51±1.0	51**±1.0
Parthenium hysterophorus (10%)	0	0	0	6±1.0	20±1.0	20**±1.0
Ageratum conyzoides (1%)	0	0	0	03±1.0	67±2.0	67*±2.0
Ageratum conyzoides (5%)	0	0	0	1±0	56±1.0	56**±1.0
Ageratum conyzoides (10%)	0	0	0	0	40±2.0	40**±2.0
Xanthium strumarium (1%)	0	0	0	16±1.0	58±2.0	65*±2.0
Xanthium strumarium (5%)	0	0	0	1±0	65±1.0	65*±1.0
Xanthium strumarium (10%)	0	0	0	1±0	53±3.0	53**±3.0

Single (*) and double asterisks (**) indicate values significantly different from control at $p=0.05$ and 0.001, respectively.

DISCUSSION

The present study evaluated several aspects of *C. asiatica* seeds focusing on germination. Freshly collected seeds had the highest viability which declined progressively as the duration of storage increased. In general, deterioration of seeds with ageing results in the loss of viability and vigour which is usually due to the alteration in moisture content, changes in biochemical composition and increased leaching of electrolytes and low molecular weight substances during imbibitions (Kalpana and Rao, 1991; Hartmann et al., 1997). Pre-treatment significantly affected the mean cumulative germination percentage for the *Centella asiatica* (Figure 2, Table 2). Exposure to GA_3 resulted in germination two weeks earlier than in control seeds, suggesting this treatment was effective in inducing metabolic activity in the embryo required for the initiation of germination process (Groot and Karssen, 1987). On a contrary, dipping seeds in warm water (60ºC) reduced nearly twice the total germination likely due to the destruction of certain enzymatic constituents present in the seed. Gill (1996) attributed the major cause of loss of viability to the scarcity of oxygen since water at high temperature has less gaseous content.

However Gill et al., (1996) reported 40% germination of seeds of *Delonix regia* when immersed in hot water for 3 minutes. Pre-treatment of seeds by 10% HNO_3 and warm water (30 ºC) also fastens the seed germination by one week earlier than the control (Table 2). This showed that these treatments enhance the metabolic activity required for germination. Jha and Jha (2002) also reported better germination of seeds of *Alysicarpus vaginalis, Desmodium triflorum* and *Axonopus compressus* after a treatment with HNO_3 for 10 min when compared to control.

Light significantly enhanced the germination of seeds of *C. asiatica*. In our study the seeds germinated significantly better in white and red light than in far-red or blue light or in the dark (Figure 3), as reported for some other species (Kiatsoonthorn and Tjitrosemito, 1992; Bell et al., 1999). Photoblastic seeds will germinate better in the open than under the forest canopy (Olatoye, 1965). In the open, solar radiation comes unhindered, moreover associated with this increase is the air and soil temperature. This best explain the exuberant growth of the seedlings of *C. asiatica* in the open land especially along the road sides and lawns at the onset of the rainy season (April-May) in Nepal. Thus, *Centella* seeds may not germinate well under a plant canopy where the FR/R ratio is high (Frankland, 1981). The mechanism of red/far-red light regulating seed germination via phytochrome system is well understood (Cone and Kendrick, 1986). The inhibitory effects of blue light and dark condition on *C. asiatica* seed germination is in agreement with previous reports (Jha and Jha, 2002). Taketay (1998) has reported germination in some photodormant *Solanum* species in the dark under the average amplitude of temperature fluctuation around 17ºC. In this study seeds of *C. asiatica* germinated in the dark considerably under the average amplitude of 20 ºC. Least germination in the dark indicates that only some *C. asiatica* seeds can germinate even below the soil depth, or without light induction. This observation suggests that photo-dormance (no white light induction) is not expressed in *Centella asiatica* seed.

Germination of *C. asiatica* decreased with an increase in salinity and was substantially inhibited at 6500 ppm NaCl (Figure 4). Maximum germination was obtained in the non-saline control. The results are in agreement with the work of Mondal et al. (1988) and Karim et al. (1992). It is also assumed that in addition to toxic effects of certain ions, higher concentration of salt reduces the water potential in the medium which hinders water absorption by germinating seeds and thus reduces germination (Maas and Nieman, 1978). It appears that a decrease in germination is related to salinity induced disturbance of metabolic process leading to increase in phenolic compounds (Ayaz et al., 2000). It is assumed that germination rate decrease with the decrease of the water movement into the seeds during imbibitions (Hadas, 1977). Salinity stress can affect seed germination through osmotic effects (Welbaum et al.,1990). Salt induced inhibition of seed germination could be attributed to osmotic stress or to specific ion toxicity (Huang and Redmann, 1995). Germination percentage also significantly decreased as the level of salinity of the medium increased (Gulzar et al., 2001; Mauromicale and Licandro, 2002).

The data on allelopathic effect of aqueous extracts of *Parthenium hysterophorus*, *Chromolaena odorata*, *Ageratum conyzoides*, and *Xanthium strumarium* exhibited significant inhibitory effects on seed germination of *C. asiatica* (Table 3). Similar findings were obtained by Oudhia (2000), who reported significant effect of *Lantana camera* leaf extract on germination of *Melilotus alba* which recorded lower germination than that of control. The minimum inhibitory effect of *C. odorata* (1%) was reported, followed by *A. conyzoides* (1%) and *X. strumarium* (1%) extracts. Inhibitory effect was extract dose-dependent, similarly to previous observations (Ballester et al., 1982; Daniel, 1999). Effects of leaf leachate of various weeds on germination, and radicle and plumule extension of field crops have also been reported earlier (Sugha, 1980; Singh et al., 1989). The inhibitory effects of studied plant species may be caused by allelopathy. These results are correlated with the findings of Kanchan and Jayachandra (1979) which found the allelopathic potential of many weed species on various field crop species in India. Inhibitory effects of leaf leachates of *P. hysterophorus, C. odorata, A.conyzoids, X. strumarium* on germination and growth of various weeds and crops have been reported earlier (Datta and Bandopadhaya, 1981, Sugha, 1980, Tefera, 2002). This could occur only when some allelochemicals present in the leaf extract prevented growth of embryo or caused the death. Further, Rice (1974) observed that many species of weeds produce toxins that are inhibitory to other weeds and often to themselves. The inhibitory effect of these species on germination has been attributed to phytotoxic chemicals released from the leaf litter and roots. Though *C. asiatica* plants grow along with weeds like *P. hysterophorus, C. odorata, X. strumarium* and *A.conyzoids* in same habitat, the inhibitory effect of these species on germination of *C. asiatica* seed may threaten its population in nature. This best explain the low density of *C. asiatica* in *P. hysterophorus* invaded site than in non invaded site (Karki, 2009).

CONCLUSIONS

Freshly harvested seeds of *C. asiatica* exhibited dormancy. The data revealed that pre-treatments with growth promoters (GA_3) positively affected *Centella asiatica* germination by reducing the time required for initiation of germination

while salinity had adverse effect on the germination of *C. asiatica* seeds. Invasive weeds *Parthenium hysterophorus, Chromolaena odorata, Artemisia* and *Xanthium* exhibited inhibitory effects on germination, which may threaten the population density of *C. asiatica* plant in nature. The information provided in this study can be used as a practical contribution for establishment of scientifically organised nursery for cultivation of medicinal plant *C. asiatica*.

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Effects of Different Light Levels on the Growth Traits and Yield of *Centella asiatica*

Anjana Devkota and Pramod Kumar Jha

Department Central of Botany, Tribhuvan University, Kathmandu, Nepal

Abstract: The growth patterns and yield of *Centella asiatica* plant under four different levels of shading 0% (full sunlight), 30, 50 and 70% of solar radiation interception were investigated.The plantlets were grown in earthen pots containing soil, sand and vermicompost (1:2:1) and submitted to different levels of shading andof solar radiation and full sunlight, as control. The results suggested that plants submitted to 30% shading showed higher plant biomass. However, the plantlets root system showed higher dry biomass under full sunlight. This information is helpful in planning cultivation of the plant.

Key words: *Centella asiatica* · Light intensity · Growth traits · Plasticity

INTRODUCTION

Light is one of the most important environmental factors affecting plant survival, growth, reproduction and distribution. Light intensity affects photosynthesis and which in turn, is related to the accumulation of organic matter and biomass. Moreover, to sustain higher photosynthetic capacity or survival, plants modify their morphology and biomass allocation at different light conditions [1]. For example, plants grown at low light intensities have higher specific leaf areas (SLA) and leaf area ratios (LAR) and lower biomass and root shoot ratios (R/S) [2, 3]. Different species, however, respond differently to light intensity. Light-demanding species are more flexible in both morphology and biomass allocation in response to light change than shade tolerant species [4, 5]. Ryser and Eek [6] suggested the adaptive phenotypic plasticity differences among species may contribute to their different abilities to occupy variable and diverse habitats in the nature. Thus, studies on the plasticity responses of plant species to light environments will contribute to our understanding of the ecological mechanism of plant distribution and assist in the development of conservation approaches to important plant species.

Centella asiatica (L) Urban is a perennial herb of the family Apiaceae. It is found throughout India and Nepal in moist places up to an altitude of 2200m (tropical to subtropical region) and also on moist stone wall or other rocky sunny areas. It is a clonal plant colonising early in the abandoned jhum (slash and burn agriculture) [7]. Observation of natural populations of *Centella asiatica* indicated extensive variation in its growth and reproductive traits. andAlthough an important traditional medicinal plant in Nepal [8]. no work on the effect of light intensities has been carried out on *Centella asiatica* plant. Therefore, the present research work has been undertaken to study the effect of light intensity on dry matter production, growth traits and chlorophyll content of *C. asiatica*. The objectives were to compare the effects of light intensity on the plant biomass, biomass allocation and morphological characters, to analyze the relative importance of these characters in response to light intensity and to find suitable light condition for cultivation purpose.

MATERIALS AND METHODS

Plants and Treatments: A pot culture experiment in a completely randomized design was established in the Botanical Garden, Central Department of Botany (CDB), Tribhuvan University, Kirtipur Kathmandu, (85°17.32'E Long and27°40.20'N Lat ,1350m asl), Nepal. Several plant cuttings of randomly sampled individual plants of *C. asiatica* were collected from same population from garden of CDB, TU, Kathmandu. The cuttings of plantlets were more or less uniform size containing four leaved condition; they were planted in earthen shallow pots filled with a mixture of field soil, sand and vermicompost(1:2:1) in green house. Altogether 160 plants; forty plants for

Corresponding Author: Anjana Devkota, Department Central of Botany, Tribhuvan University, Kathmandu, Nepal
Tel: 977-1-4331322

each treatment were planted separately for experiment. Planting was done in October 2007. After two weeks they were then transferred to three distinct shading levels (30, 50 and 70%) and full sunlight as control. The light was controlled by different layers of nylon-net shade placed 2 m above ground. The plantlets were irrigated at regular periods depending on the weather and soil moisture status. Each treatment was repeated twice.

Measurements and Calculation: Data on yield and morphological traits were recorded in April 2008. All plants per replication were used for the observations. Sixteen quantitative traits pertaining to plant morphology and yield were measured. Ninety mature leaves per treatment were measured for petiole length (PL) and specific leaf area (SLA). Petiole length, length and width of leaves were measured in fresh leaves. Then these leaves were oven dried (60°C, 48 h) and mass of each leaf was weighed in electric balance (0.001g). Length and width of leaves were measured and multiplied by conversion factor following Zobel *et al.* [9] for determination of leaf area. SLA was calculated as the ratio of leaf area and dry mass.

Leaf nitrogen (N) content was determined by modified micro Kjeldahl method following the procedure described by Horneck and Miller [10]. Leaf N content was determined in twenty samples from each treatment. Chlorophyll a, Chlorophyll b and total chlorophyll content was determined following the method of Arnon [11] in five samples from each replication.

Number of nodes (NND) occurring along each primary branch were noted. Internodal lengths (IND) were also measured on primary branches arising from mature rosettes. The number of leaves (NLN) and primary branches (NBN) arising from it was also scored. Inflorescences were measured for flower pedicel length (FPL) and total number of flowers per mature rosette. Dry mass of individual plant per replication was obtained after harvest. R/S ratio was calculated by dividing root mass with shoot ass.

RESULTS

Growth and Morphological Characters: All the measured traits of leaves varied significantly with light intensity (Table1). Among the leaf traits, the extent of variation was the highest in specific leaf area (CV=124, Table 1). Average number of leaves was 18.62 per ramet. There was significant difference in leaf number among the treatments with the highest number of leaves in plants grown in 30% shade (Table 1). Petiole length ranged from 2.4 cm at full sunlight to 4.63 cm at 70% shading. All treatments differed significantly ($p< 0.001$) in petiole length. Regarding the internode length, it was significantly longer ($p< 0.001$) in plants grown at 70% shading (Table1). Specific leaf area ranged from 195.16 cm^2/g at full sunlight to 1536.2 cm^2/g at 70% shading (average 892.86 cm^2/g). The difference in specific leaf area (SLA) among the treatments was significant ($p=0.002$) (Table 1). Regarding leaf chlorophyll concentration, an increment in these photoreceptors was observed with the increase of shading (Fig. 1), reaching the highest values in plants cultivated under 70% shading. There was significant difference ($p=0.001$) in leaf N concentration ranging from 1.73% at full light to 2.66% at 70% shading (Table 1). Number of primary branches per ramet also differed significantly ($p<0.001$) among treatments, a higher number being in treatment with 30% shading (Table 1). The number of flower per ramet ranged from 17.85 in plants grown in full sunlight to 0.94 in plants grown in 70 % shading condition.

Table 1: Growth traits and yield of *Centella asiatica* in different light conditions .For each parameter significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, $\alpha = 0.05$). F and P values were obtained by one way analysis of variance (ANOVA).

Attributes[a]	Full light	30%Shade	50% Shade	70% Shade	Mean	CV (×100%)	F value	P value[b]
No. of leaves[$]	20.26±9.6a	22.65±3.82b	16.6±8.41ab	12.57±4.64a	18.02±7.5	0.45	4.456	0.006
SLA(cm^2/g)*	195.16±30.29a	967.93±1092b	873.2±954.71b	1536.2±1.46b	892.86±1114.7	1.24	5.429	0.002
Petiole length (cm)*	2.47±0.6b	2.55±0.51a	4.55±0.91a	4.63±0.77b	3.55±1.26	0.35	54.173	0.000
Leaf N (%) #	1.73±0.23a	2.38±0.5b	1.81±0.28a	2.66±0.22c	2.14±0.5	0.23.	35.311	0.000
Number of branch[$]	3.78±2.37b	5.55±1.46c	3.55±2.06b	2±0.66a	3.74±2.14	0.57	13.218	0.000
Number of nodes[$]	2.84±1.06b	7.62±0.805c	3.2±0.95b	2±0.66a	3.95±2.37	0.6	160.182	0.000
Length of internode(cm)[$]	0.87±0.44a	4.92±0.69b	4.93±0.68b	5.1±0.84b	3.93±1.92	0.48	182.726	0.000
Peduncle length(cm)[$]	0.11±0.26a	0.59±0.06b	0.58±0.068b	2c	0.83±0.72	0.86	689.267	0.000
Number of flower per node[$]	17.85±11.52c	10.65±11.28b	9.63±9.77b	0.94±2.24a	9.88±11.14	1.12	10.220	0.000

[a] Sample size(n) for each treatment: *n=90 ;$=40 and# n=20;b Bold number indicates significant difference among the mean, ± Standard deviation, CV= Coefficient of Variance

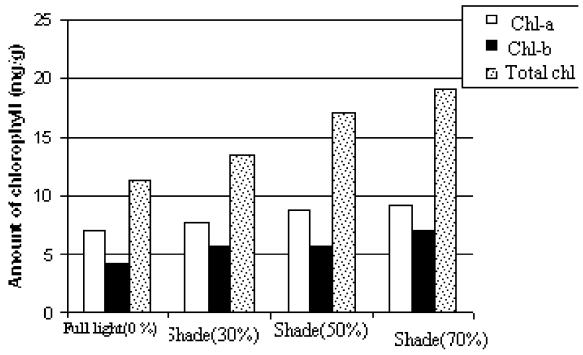

Fig. 1: Mean values of chlorophyll concentration of *Centella asiatica* submitted to different levels of shading

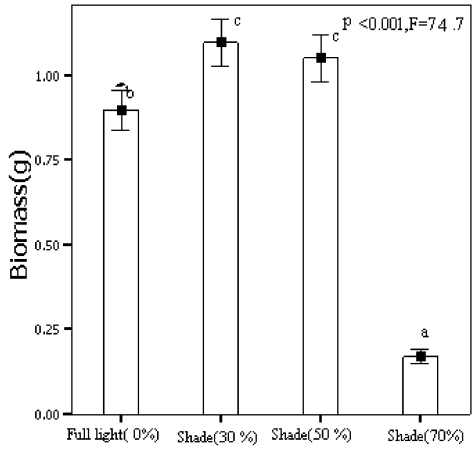

Fig. 2: Mean values of Biomass of individual plant of *Centella asiatica* submitted to different levels of shading (Means followed by the same letter do not differ by the Duncan Test at 5%)

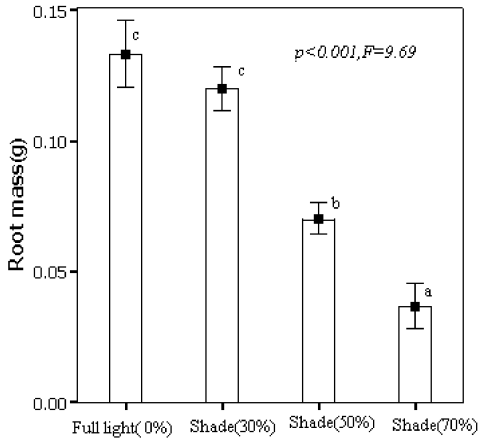

Fig. 3: Mean values of root mass of *Centella asiatica* submitted to different levels of shading (Means followed by the same letter do not differ by the Duncan Test at 5%)

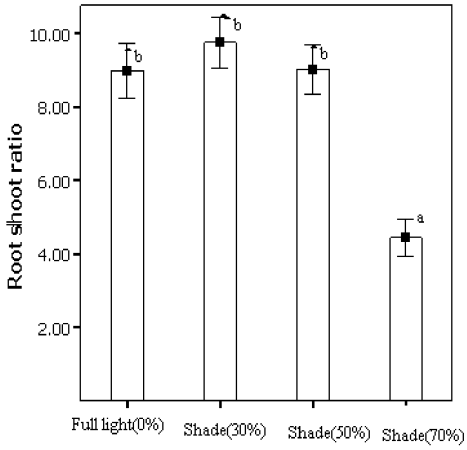

Fig. 4: Mean values of root / shoot ratio of *Centella asiatica* submitted to different levels of shading (Means followed by the same letter do not differ by the Duncan Test at 5%)

Biomass Allocation and R/s Ratio: There was significant effect of light intensity on biomass allocation of *C. asiatica* plant. It was significantly higher in partially shade (30% shade) than in other treatment (Fig.2, $p<0.001$). Less shaded plants tended to concentrate the dry weight in roots in relation to more shaded plants (Fig. 3). The R/S ratio of the *Centella asiatica* plant was significantly affected by light intensity as it was highest in full light (Fig. 4, $p<0.001$).

DISCUSSION

Growth and Morphological Characters: Light intensity had significant influence on growth and morphological characters. The number of leaves under full sunlight was, on average twice that of shaded plants (Table 1). Some other tropical species showed the similar response, as in *C.asiatica*, which increased twice the number of leaves growing in gaps when compared to shade grown plants [12].

The shaded plants (50% to 70%) produced larger leaves, longer petiole and internode length, in order to capture more light, probably because of a shade-avoidance mechanism [13] which resulted in decreasing the flower buds, showing that plants grown in lower light conditions tended more on vegetative part growth like leaf area, petiole length and internode length rather than reproductive growth [14].

The significantly lower specific leaf area in high light *C.asiatica* suggests leaf anatomical differences brought about by low quantum flux density [15] and reflects a

strategy to increase this species competitive ability under low light through an increase in leaf area. The highest values for SLA in more shaded treatment could be due to the increase in leaf area and a reduction in thickness caused by shading. andLeaves in the sun are usually thicker than those growing in the shade [16]. An increase in SLA is a common response observed in plants under low light conditions [17-19] and is usually associated with extra layers of mesophyll cells [20].

Leaf chlorophyll was found to increasing with increasing of shading (Fig. 1), up to 70%. Leaf chlorophyll levels are controlled by light [21]. In elevated radiation intensities, chlorophyll molecules are susceptible to photo oxidation and the equilibrium is reached in lower radiation levels [22]. This was the reason for having higher chlorophyll levels in shaded leaves than leaves grown under full sunlight. Similar results were obtained by Alvarenga *et al* [23], in seedlings of *Guarea guidonia*.There was significant positive correlation (r^2=0.325, p=0.003) of leaf N with SLA. It was the reason for having high nitrogen content in plants grown in more shaded site. This response followed the same pattern reported by other studies with tropical species [20].

Biomass Allocation and R/s Ratio: In the present study, at plantlets grown under full sunlight compared with those grown under shading, a higher increase in root dry weight was verified in relation to the aerial part (Fig. 4). A reduction in specific leaf area and higher translocation of photoassimilates to roots (Fig. 3 and 4; Table 1) were observed. Young plants of *Garcinia mangostana* showed a similar behaviour, reduced leaf area and higher dry weight translocation to root system under decreasing shading conditions [24]. As suggested elsewhere [20], the lowest allocation to roots under low light conditions is known to be maximized in sun-loving plants and probably reflects a response to attributes that improve carbon gain under reduced irradiance such as an increase in SLA, or that reflects a light seeking strategy such as an increase in petiole length and internode length. A common response to shade reported in many studies is a reduced allocation to roots [20, 25, 26].

CONCLUSION

There was significant variation in many vegetative growth traits and yield of *C. asiatica* along different levels of light intensity. The results also suggest that *Centella asiatica* plant showed a better development when exposed to shading of 30%. This information can be used in planning cultivation of the plant.

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Research

Variation in the active constituent contents in *Centella asiatica* grown in different habitats in Nepal

Anjana Devkota[1]*, Stefano Dall' Acqua[2], Pramod Kumar Jha[1] and Gabbriella Innocenti[2]

[1]Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal; [2]Department of Pharmaceutical Sciences, University of Padova, Padova, Italy

Abstract

Centella asiatica is an important medicinal plant of subtropical to tropical region. It grows widely, in different habitats. In Nepal, it is distributed at an altitudinal range of 96-2200 m above sea level. A comparative quantitative analysis of chemical constituents in *Centella asiatica* samples collected from three different habitats in Nepal was carried out by HPLC to evaluate the variability in the important constituents. There was marked variability in asiaticoside, asiatic acid and quercetin 3-*O*-glucuronide content among the samples collected from different habitats. Samples collected from open agricultural land showed the highest asiaticoside (1.91%), asiatic acid (0.13%) and quercetin 3-*O*-glucuronide (0.35%) content. Therefore, open land is preferable for plantation of this species for high yield of secondary metabolites.

Key-words: Asiaticoside, asiatic acid, *Centella asiatica*, HPLC, medicinal plants, quercetin 3-*O*-glucuronide.

Introduction

Centella asiatica (L.) Urban, also known as 'gotu kola' or 'Indian pennywort', is a tropical medicinal plant with a long history of therapeutic use, particularly in dermal disorders, venous insufficiency and microangiopathy. Reports from various places have revealed that *C. asiatica* has been used for wound healing (Shukla *et al*. 1999), memory improvement, bronchitis, asthma, dysentery, leucorrhoea, kidney trouble, anti-allergic and anticancer purposes, curing leucorrhoea and toxic fever (Kan 1986). Clinical trials have also shown that it can help those with chronic venous insufficiency (Brinkhaus *et al*. 2000). *Centella asiatic*a mainly contains asiatic acid, madecassic acid, terminolic acid, vanillic acid, succinic acid, asiaticoside, asiaticoside-B, madecassoside, asiaticodiglycoside. The main active components of the plant are believed to be triterpenoids. Several studies have revealed the triterpenoid derivatives of *Centella asiatica* using different techniques (Diallo *et al*. 1991; Du *et al*. 2004). A HPLC method was set for quantitative determination of six triterpenes in *Centella asiatica* extracts and commercial products by Schaneberg *et al*. (2003). Recently, Devkota *et al*. (2010) obtained data about the variations in secondary metabolite in different geographical areas of Nepal selecting high producing triterpene plants for possible cultivation. In this study, we collected plant material from three different habitats, *viz*. open agricultural land, open grassland and shady grassland to analyze the influence of habitats on the eight main chemical constituents of *Centella asiatica*. We used an ELSD detector because of the poor UV absorption of the triterpene nucleus.

*Corresponding author, email address: devkotaa@gmail.com

Materials and Method

PLANT MATERIALS

Plant samples (aerial parts) ($n = 38$) of *Centella asiatica* were collected from different habitats in Nepal: (a) open grassland (where grazing pressure was high and vegetation was dense);

(b) partially shade grassland (where vegetation was dense, and grazing was prohibited) and (c) open agricultural land (moderately grazed open land, receiving full sunlight and with sparse vegetation). Samples were collected in April-May 2007 and shade dried.

COLLECTION AND ANALYSIS OF SOIL SAMPLES

Soil samples (n = 10), from each habitat, was collected from the root zone at the time of collection of plant samples. Then, samples were air dried and used for analysis. Soil organic carbon was determined by the Walkley Black rapid titration method and total N by micro-Kjeldahl method (Jackson 1958).

CHEMICALS AND REAGENTS

HPLC grade acetonitrile, methanol and formic acid were purchased from Carlo Erba Italy. HPLC grade water was prepared by filtering nanopure water through a 45 µm membrane filter (MilliQ).

REFERENCE SAMPLE

Asiaticoside, kaempferol, quercetin, rosmarinic and chicoric acids were purchased from Phytolab GmbH, Germany. Chlorogenic acid was purchased from Sigma Aldrich. Quercetin 3-O-glucuronide was purified from the extracts of *C. asiatica* as described by Satake *et al.* (2007).

PLANT SAMPLE PREPARATION

Approximately 100 mg of ground plant material (whole plant parts) was placed into a 15 ml falcon tube (screw capped polypropylene centrifuge tube) and extracted three times with 5.0 ml of methanol by sonication. The extract was centrifuged 5 minute in 3000 rpm and the supernatants were combined to a 25 ml volumetric flask by pipette, diluted to final volume with methanol and mixed thoroughly. All samples were filtered through a 0.45 µPTFE syringe filter before the injection in HPLC.

HPLC CONDITIONS

Instrumentation consisted of an Agilent 1100 series liquid chromatograph equipped with Agilent 1100 Diode Array (DAD) and SEDEX LT60 Evaporative Light Scattering Detectors (ELSD). An Agilent XDB-C-18 reverse phase column (25×4.6 mm, 4.6 µm) was used as stationary phase. The gradient elution program, with aqueous formic acid (0.1%) (A) and acetonitrile (B), was: 0-8.5 min, linear gradient from 12 to 26 % B; 8.5-11 min, isocratic conditions at 26 % B; 11-16 min, linear gradient from 26 to 40 % B; 16-45 min, linear gradient from 40 to 50 % B; 45-50 min, linear gradient from 50 to 100 % B. Flow rate was 1 mL/min and injection volume 20 µL. The ELSD detector temperature was 50°C, nitrogen pressure 2.2 bars, and the gain level 10 arbitrary units (a.u.). For the sample analyses, a gradient elution was used; using an eluent A: Acetonitrile, B: Methanol, C: water with 0.1% HCOOH. Gradient is presented in Table 1.

Table 1. Gradient scheme used in HPLC analyses.

Time (Minutes)	Solvent (ml)		
	Acetonitrile	Methanol	Water with 0.1% HCOOH
0	10	2	88
10	26	4	70
22	25	5	70
27	30	0	70

Calibration curves were obtained by preparing standard solutions, as listed in Table 2. Asiaticoside and asiatic acid were determined with the ELSD detector. Chlorogenic, chicoric and rosmarinic acids were determined with the DAD at 330 nm, and at 350 nm for kaempferol, quercetin and quercetin 3-*O*-glucuronide. HPLC chromatogram of the standard compounds is reported in Fig. 1.

DATA ANALYSIS

The data were analyzed to assess the difference in measured attributes among the habitats by one way analysis of variance (ANOVA) and the Duncan's homogeneity test using Statistical Package for Social Science, version 11.5 (SPSS 2002).

Results

HPLC analysis revealed marked variability in the analyzed bioactive components among the samples collected from different habitats (Table 3). Content of these phytochemicals varied greatly in different habitats depending upon the nature of chemical constituents. The amount of most of the analyzed chemical constituents was higher in open agricultural land than in other habitat types. However, the difference was statistically significant for asiaticoside, asiatic acid, quercetin 3-*O*-glucuronide, kaempferol and rosmarinic acid content ($p < 0.05$) (Table 3). Samples collected from open agricultural land showed the highest asiaticoside (1.91%), asiatic acid

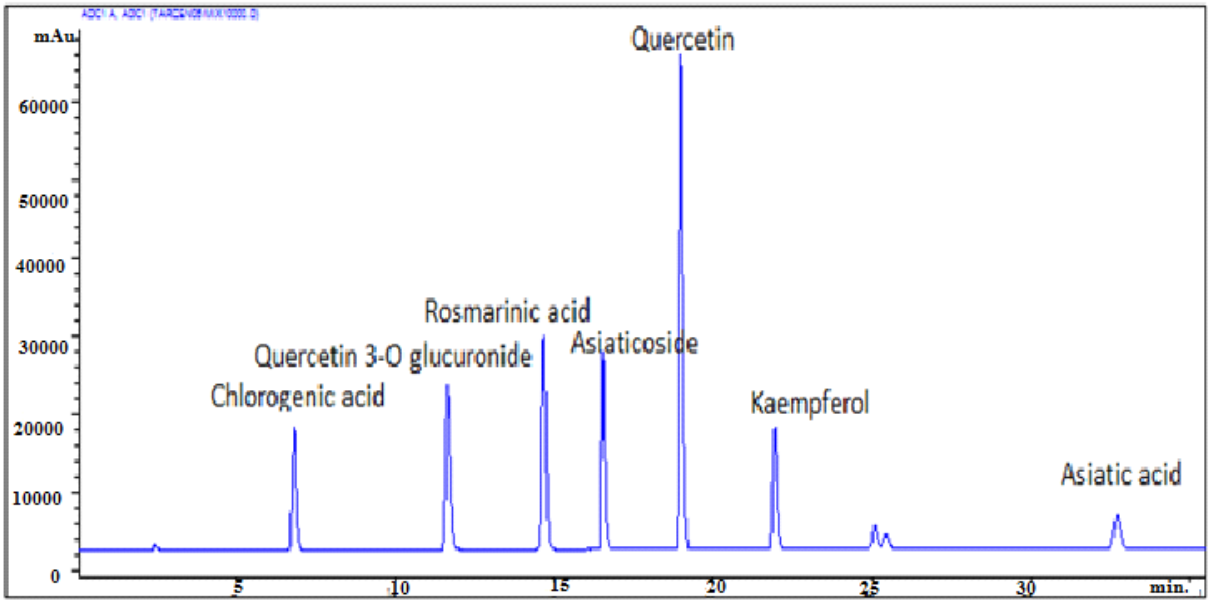

Figure 1. HPLC chromatogram of the standards used for the quantitative determination.

Table 2. Concentration ranges and calibration curves for the analyzed secondary metabolites.

Analyte	Concentration (µg/mL)	Regression curve*	R^2 (n = 6)	LOD (µg/mL)	LOQ (µg/mL)
Asiatic acid	4.250 - 100.00	Log y = 0.608 Log x – 1.880	0.9976	1.04	3.46
Asiaticoside	5.620 - 140.50	Log y = 0.588 Log x – 1.872	0.9989	1.02	3.40
Chicoric acid	0.732 - 73.17	y = 0.0127 x – 0.0886	0.9998	0.29	0.97
Chlorogenic acid	0.685 - 97.53	y = 0.0168 x – 0.2456	0.9989	0.40	1.33
Rosmarinic acid	0.766 - 113.00	y = 0.0147 x + 0.1534	0.9995	0.35	1.17
Quercetin	0.840 - 84.03	y = 0.0086 x + 0.9724	0.9982	0.21	0.70
Quercetin 3-O-glucuronide	1.300 - 130.00	y = 0.0393 x – 0.0249	0.9983	0.10	0.33
Kaempferol	0.660 - 65.97	y = 0.0136 x + 0.391	0.9997	0.19	0.63

*x = peak area; y = concentration of analyte (ìg/mL).

LOD = Limit of Detection, LOQ = Limit of Quantification.

Table 3. Phytochemical constituents of *Centella asiatica* from different habitats. For each parameter, significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, *á* = 0.05). F and P values were obtained by one way analysis of variance (ANOVA).

Chemical constituents	Partially shade grassland (n=12)	Open grassland (n=12)	Open agricultural land (n=14)	Mean	F value	P value
Asiaticoside	$1.41^{a} \pm 1.10$	$1.71^{b} \pm 1.03$	$1.91^{b} \pm 1.03$	1.68 ± 1.60	10.334	0.030
Asiatic acid	$0.08^{a} \pm 0.01$	$0.07^{a} \pm 0.02$	$0.13^{b} \pm 0.11$	0.09 ± 0.02	0.604	0.012
Chicoric acid	$0.05^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	$0.06^{a} \pm 0.01$	0.05 ± 0.10	0.603	0.321
Chlorogenic acid	$0.24^{a} \pm 0.18$	$0.23^{a} \pm 0.12$	$0.26^{a} \pm 0.08$	0.25 ± 0.13	0.117	0.190
Quercetin	$0.35^{a} \pm 0.10$	$0.38^{b} \pm 0.02$	$0.38^{b} \pm 0.00$	0.37 ± 0.06	1.154	0.198
Quercetin 3-*O*-glucuronide	$0.17^{a} \pm 0.00$	$0.28^{b} \pm 0.01$	$0.35^{c} \pm 0.01$	0.25 ± 0.01	17.24	0.025
Kaempferol	$0.03^{a} \pm 0.11$	$0.35^{a} \pm 0.10$	$0.39^{b} \pm 0.06$	0.36 ± 0.09	7.301	0.042
Rosmarinic acid	$0.18^{a} \pm 0.07$	$0.16^{a} \pm 0.08$	$0.18^{b} \pm 0.17$	0.15 ± 0.12	1.301	0.032

Table 4. Nutrient content in soils from different habitats. For each parameter, significant difference between mean among the sites are indicated by different letters (Duncan homogeneity test, $\acute{a} = 0.05$). F and P values were obtained by one way ANOVA.

Habitats	Soil Nitrogen (%)	Soil Organic Carbon (%)	Soil Organic Matter (%)
Partially shade grassland	$0.24^{a} \pm 0.12$	$2.34^{a} \pm 0.98$	$4.23^{a} \pm 1.73$
Open grassland	$0.13^{b} \pm 0.05$	$1.16^{b} \pm 0.51$	$2.03^{b} \pm 0.89$
Open Agricultural Land	$0.13^{b} \pm 0.23$	$1.52^{b} \pm 0.47$	$2.58^{b} \pm 0.82$
F value	16.72	9.23	22.13
P value	<0.001	<0.001	<0.001

(0.13%) and quercetin 3-*O*-glucuronide (0.35%) content. Asiaticoside was the most dominant constituent (mean 1.68% dw); its value ranged from 1.41% in shady grassland to 1.91% in open agricultural land (Table 3). The content of chicoric acid, chlorogenic acid and quercetin did not differ significantly among the habitats.

There was significant difference in nutrients in soil collected from different habitat (Table 4). Soil N was higher in open agricultural land than in other sites.

Discussion

Present study showed that habitat factor may impose significant impact on accumulation of important bioactive components in plants. Significantly higher amount of phytochemical constituents was measured in samples collected from open agricultural land and least from shady grassland. The plants growing on open agricultural land are possibly under stress due to direct sunlight and less availability of moisture. Perhaps due to increased solar radiation and temperature, the plants produced more secondary compounds in relation to the adaptation mechanism. Odabas *et al.* (2009) hypothesized that the high photosynthetic activity under high light intensity resulted on increased amount of carbon assimilation and enhanced the concentration of carbon-rich secondary metabolites in leaf tissues. In this study, soil from open agricultural land contained relatively low nutrient (C and N) as compared to shady habitat (Table 4). Nutrient stress generally reduces growth more than it reduces photosynthesis per second (McKey 1979) and thus, it has been argued that the expected surplus of carbon can lead to an accumulation of carbon-based secondary substances under such circumstances (Bryant *et al.* 1983). This might be the reason for having low amount of secondary metabolites in shady grassland with relatively high soil nutrient contents.

Significant difference in contents of active constituent have been observed in samples of *C. asiatica* originating from different countries, such as India and Madagascar (Das and Mallick 1991; Rouillard-Guellec *et al.* 1997). Comparative study by Das and Mallick (1991), in 10 ecotypes of *Centella asiatica* from different regions of India, showed a correlation between genomic diversity and asiaticoside content. In present study, mean asiaticoside content in samples of *Centella asiatica* was 1.68% (dw). It has been shown that leaves of *C. asiatica* from Nepal contain 4 to 10 time's higher concentration of asiaticoside than those from India (Rouillard-Guellec *et al.* 1997). Low quantity of asiatic acid and chicoric acids were recorded in all analyzed samples (Table 3). Foo and Porter (1980) have reported that the compounds with lower molecular weight are usually present in plant tissue in relatively low concentrations compared to that of larger polymers. Generally, all *C. asiatica* samples showed relatively higher amount of asiaticoside than asiatic acid. This is in accordance with large amount of triterpene glycosides and trace of triterpenic acids from plants of Thailand, Costa Rica and Bahamas (Booncong 1989). However, high asiatic acid content was reported in *C. asiatica* of Malaysia (Pick Kiong 2004). The observation in this study is in agreement with the statement by many researchers that *C. asiatica* collected from different locations produced different amount of triterpenes. Apart from the environment, climate and soil condition, the method of extraction could also be a contributing factor for the diverse compounds in *C. asiatica* from various locations (Booncong 1989).

Conclusion

Different habitats have significant effect on accumulation of active phytochemicals in *C. asiatica*. Open land is preferable for plantation of this species for high yield of secondary metabolites, especially the marker compound (asiaticoside).

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Influence of water stress on growth and yield of *Centella asiatica***

A. Devkota and P.K. Jha*

Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

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A b s t r a c t. The influence of water stress on growth and yield of *Centella asiatica*, a traditional medicinal herb of Nepal, was carried out in a pot experiment. Variation in different growth traits of *Centella asiatica* was investigated using vegetative clones of one population. The plantlets were grown in earthen pots containing soil, sand and vermicompost and treated with different levels of water stress (30, 70, 100, and 125% of pot capacity by mass). The experimental design was completely randomized and each treatment was composed of forty plants. An array of vegetative traits including: number of leaves, petiole length, specific leaf area, number of primary branches, and plant biomass was examined. Growth traits such as root length, leaf area and number of flowers per ramet demonstrated significant variation in response to water stress. The results suggested that plants irrigated to 100% pot water capacity showed highest growth and plant biomass production.

K e y w o r d s: *Centella asiatica*, growth, water stress, yield

INTRODUCTION

Centella asiatica L. Urban is one of the most important traditional medicinal herbs found in tropical to subtropical region of Nepal. This plant is widely used by locals as traditional medicine for the reduction of uric acid in blood, for the treatment of high blood pressure, and also as a memory enhancer as well as blood purifier (Devkota and Jha, 2008). Limited water supply is one of the most important environmental factors affecting productivity of crops and medicinal plants (Rahman *et al.*, 2004). Physiological changes in plants, which occur in response to moisture deficiency, decrease photosynthesis and respiration (Sarker *et al.,* 2005), and, as a result, overall production of the crop is decreased. Greater soil water stress decreased plant height and total fresh and dry mass of *Satureja hortensis* – a traditional medicinal herb of Iran (Baher *et al.,* 2002). It was shown that the number of stems per plant and plant dry mass was negatively related to water stress in a perennial weed *Eragrostis curvula* (Colom and Vazzana, 2002). Although the effects of water stress on growth and yield of different crops have been studied during the last years (Ahmad *et al.*, 2003; Kumaga *et al.*, 2003; Rahman *et al.*, 2004; Tahir and Mehdi, 2001), no such work has been done to study the effects of water stress on the medicinal on herb *Centella asiatica* in Nepal.

The objective of the paper was to investigate the possible effect of water stress on growth traits and yield of *Centella asiatica*.

MATERIALS AND METHODS

A pot culture experiment in a completely randomized design was established in the Botanical Garden, Central Department of Botany (CDB), Tribhuvan University, Kirtipur, Kathmandu, Nepal (85º17'32"E, 27º40'20"N, 1 350 m a.s.l.). The growth medium utilized in the experiment was comprised of clay (garden soil), horticultural sand, and vermicompost in the ratio of 1:2:1.Variation in different growth traits of *Centella asiatica* was investigated using vegetative clones of plants from one population from the garden of CDB, TU, Kathmandu. Besides this, no fertilizer was added during the experimental period. Plantlets were collected from a mostly dense site within a 5×5 m area, to reduce the probability of genetic differences among the plantlets. The cuttings of plantlets were planted in earthen shallow pots (mean diameter: 20 cm), one cutting per pot, which were filled with the growth medium as described above. The pots were then transferred to a sort of green house at CDB, TU Kirtipur. The temperature of the green house was slightly

*Corresponding author's e-mail: devkotaa@gmail.com

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higher than outer environment (+3 to 5°C more). Planting was carried out in October, 2007 and four different levels of water stress were applied (125% – surplus water, l00, 70, and 30% of pot capacity by mass; T1, T2, T3, T4, recpectively). Forty plants for each treatment were planted. A total of 160 plants were planted separately for the experiment. The first treatment (125%) was applied at the beginning of the experiment. Drainage was prevented in those pots to represent the excessive water treatment by closing the hole lying at the bottom of each earthen pot. The pots were weighed every two days and water was added using a beaker to compensate for the water loss by evapotranspiration. The soil moisture in the other treatments was maintained every two days at 100, 70, and 30% of pot water capacity (PWC). Weeding was done as required. All pots and treatments were rotated each week to counter any positional effects of pots within treatments. Some of the experimental plants were damaged by insects. As a result, only thirty plants per treatment were randomly selected and were used for the observations. Data on yield and morphological traits were recorded in April, 2008.This experiment was repeated in the 2009 growing season.

Growth traits pertaining to plant morphology and herb yield were recorded. Ninety mature leaves per treatment *ie* three leaves per plant were measured for petiole length (PL) and specific leaf area (SLA). After the length and width of each leaf was measured, leaves were oven dried (60 ºC, 48 h) and the mass of each leaf was weighed with an electric balance (to 0.001 g). The length and width of each leaf was multiplied by a conversion factor to calculate total leaf surface area (Zobel *et al.,* 1987). Specific leaf area was obtained as the ratio of total leaf surface area and dry mass of each leaf.

Leaf nitrogen (N) content was determined by a modified micro-Kjeldahl method (Horneck and Miller, 1998). For leaf nitrogen analysis, leaves collected from 20 individuals per treatment were taken. Five samples from each treatment were taken for measurement of chlorophyll-*a*, chlorophyll-*b*, and total chlorophyll content (Arnon, 1949).

Number of nodes (NNB) occurring along each primary branch were noted. Internodal lengths (IND) were also measured on primary branches arising from mature rosettes. The number of leaves (NLN) and primary branches (NBN) arising from each rosette were also scored. The total number of flowers per mature rosette was also noted. The total fresh and dry masses of each were measured after harvest, and the total moisture content (MC) of plant was measured. Root length of each individual was measured. Whole soil of pot together with plant was dropped out from it to prevent destruction of root parts, while uprooting roots only.

The significance of the difference in measured attributes among the treatments was tested by one way analysis of variance (ANOVA). The amount of variation in the parameters in response to the treatments was assessed by calculating the coefficient of variation (CV) computed as the percentage ratio of standard deviation of the mean. The treatment types were also compared by Duncan multiple range tests. The multiple range tests allow comparison of the pairs of treatment for each attribute. SPSS version 11.5 (2002) was used for all statistical analysis.

RESULTS

All morphological traits were affected significantly by the treatments (Tables 1 and 2). Leaf number per ramet ranged from 5.40 to 11.11 (average 7.50). All treatments differed (p<0.001) significantly in leaf number per ramet (Table 1). An effect of different water stress treatments on leaf morphological characters, like petiole length and specific leaf area, was noted during the experimental period. Among the leaf traits the extent of variation was the highest in leaf area (CV = 45.78) and lowest in number of leaves per ramet (CV = 12.29). The longest petiole length (5.67 cm) was observed at 100% PWC and the shortest (2.05 cm) at 30%. Similarly the highest value of SLA (417.3 $cm^2\ g^{-1}$) was obtained at 30% PWC and the lowest (136.5 $cm^2\ g^{-1}$) at 70% PWC. Leaf N content ranged from 1.4 to 2.54%

T a b l e 1. Growth, yield and some chemical characters of *Centella asiatica* leaves in different water stress condition (average from 2008-2009)

Attributes[a]	(T1)	(T2)	(T3)	(T4)	Mean	CV	F value	P value
Number of leaves per ramet[$]	6.90a	11.11b	6.60a	5.40a	7.50	12.29	10.27	0.001
Petiole length (cm)[#]	4.12c	5.67c	3.21b	2.05a	3.54	29.09	31.4	0.001
Dry mass of leaf (g)[#]	0.023b	0.021b	0.027c	0.012a	0.021	18.33	21.38	0.000
Area of leaf (cm^2)[#]	5.27c	5.1c	3.73b	2.46a	4.15	45.78	50.257	0.000
Specific leaf area($cm^2\ g^{-1}$)[#]	217a	261a	136.5a	417.3b	257.95	19.23	4.3	0.003
Leaf N (% d.m.)*	1.68a	2.25b	2.54b	1.4a	1.77	27.11	21.19	0.000
Total chlorophyll[*] (mg g^{-1})[+]	10.15a	25.75b	25.43b	9.76a	17.27	13.27	9740.612	0.000

For each parameter significant difference between mean among different treatments are indicated by different letters (Duncan test, $\alpha = 0.05$). Means marked by the same letter are not significantly differed, F and P values were obtained by one way analysis of variance.
[a]Sample quantity (n) for each treatment: [#] n = 90;* n = 20; [+]n = 5; [$] n = 30.

T a b l e 2. Growth traits and yield of *Centella asiatica* plant in different water stress condition (average from 2008-2009)

Attributes[a]	(T1)	(T2)	(T3)	(T4)	Mean	CV	F value	P value
Number of primary branch per ramet[$]	5c	5.5c	2.8b	1.3a	3.65	21.34	37.92	0.000
Number of nodes[$]	2.62b	4.44c	3.03b	1.66c	2.99	51.5	24.13	0.001
Length of internode (cm) [$]	5.077b	5.21b	3.84a	3.8a	4.52	19.24	46.91	0.001
Number of flower per node[$]	5.11b	17.66d	6.37c	0.0a	7.28	80.9	58.5	0.001
Root Length[$] (cm)	8.2a	8.82ab	7.1a	10.56c	8.58	15.64	7.5	0.001
Dry mass of individual plant[$] (g)	0.31a	1.44c	0.83b	0.23a	0.67	24.56	46.56	0.000

For each parameter significant difference between mean among different treatments are indicated by different letters (Duncan test, α = 0.05). Means marked by the same letter are not significantly differed. F and P values were obtained by one way analysis of variance (ANOVA). Explanations as in Table 1.

(average 1.77%). All treatments differed ($p < 0.001$) significantly in leaf N content. Total chlorophyll content ranged from 9.76 to 25.75 mg g^{-1} with an average value of 17.27 mg g^{-1}. There was significant difference in total chlorophyll content among treatments ($p<0.001$) (Table 1).

Root length of plants in each treatment was highly significant ($p = 0.003$) (Table 2). Longest root length (10.56 cm) was observed with the plant at 30% PWC. There was significant difference ($p < 0.001$ in number of primary branches and number and length of stolons among the treatments. A significant effect of different water stress treatments on number of flowers per plant was noted during study period. The highest number of flowers (17.66 flowers per ramet) was obtained at 100 % and the least number of flowers was at 30% PWC. A significant effect of different water stress treatments on yield of *C. asiatica* plant was also found during the study period. The yield of plants in the different water stress treatments revealed that the highest drymass (1.44 g $ramet^{-1}$) was obtained at 100% and lowest (0.23 g $ramet^{-1}$) at 30% PWC.

DISCUSSION

Drought stress is one of the important growth limiting factors of *Centella asiatica*, which decreases plant growth during the vegetative stage. We observed effects of water stress upon most vegetative traits. An effect of different water stress treatments on leaf morphological characters, such as petiole length and specific leaf area, was noted during the experimental period. The largest leaf area was recorded at 125% PWC treatment. Leaf area decreased with increased water stress, and the minimum leaf area was observed in the 30% PWC treatment. The smaller leaf area transpires less water and this reduction in leaf area can therefore be considered a first line of defence against drought. This reduction in leaf area under water stress is similar to studies on *Vigna subterranea* (Mwale *et al*., 2007; Vurayai *et al*., 2011). Number of leaves per ramet also lowest at 30% PWC. These results are consistent with the work of Khalil *et al.* (2010) and Moeini *et al.* (2006) in *Ocimum basilicum* plant. Reduction of leaf area by severe water stress can be considered as an adaptive mechanism, which helps to reduce water loss from the plant (Turk and Hall, 1980).

Leaf N and chlorophyll content were lowest in plants grown in soil having 30% PWC. The lowest value of leaf N and chlorophyll of *C. asiatica* at T4 (PWC 30%) was due to low moisture content in the soil. Low moisture content in soil causes an inability of plants to get all available nutrients in the soil, and consequently, it causes low levels of water and nutrients in a plant. The decrease of chlorophyll content in plants growing at T4 (PWC 30%) might be most probably related to the decrease of the water content of plants (43.75%). Wang and Nii (2000) stated that the decrease of chlorophyll *a* content is highly related to the decrease of water content in plant leaves. Root growth was also affected by water stress. Under extreme water stress, the roots more easily penetrated the soil, possibly providing greater access to the little water available and promoting production as a result. This might be the reason for having the longest roots at 30% PWC.

Dry matter content of plants differed significantly ($p = 0.001$) among water stress treatment. The highest value of dry mass was observed in the 100% PWC followed by 70% pot treatment. The total dry masss of plant decreased with exposure to high water stress (30%) or excessive water (125%). This could be the result of a reduction in chloro- phyll content and, consequently, photosynthesis efficiency, as reported by Khalid (2006) in *Ocimum* sp., Said-Al Ahl *et al.* (2009) *in Origanum vulgare* and Khalil *et al.* (2010) in *Ocimum basilicum* respectively. This result could be due to that one of the first signs of water shortage was the decrease of turgor which resulted in decrease in growth and development of cell especially in leaves (Alishah *et al.*, 2006). When the leaf

level decreases, the light draw decreases and the total capacity of photosynthesis decreases, so plant growth became less and plant performance decreases which leads also to the decrease in dry matter production. This result agrees with findings of Alishah *et al.* (2006) in *Ocimum basilicum* and Khalid (2006) in *Ocimum* sp., respectively.

Reduction of fresh and dry matter content and yield of a medicinal plant, *Mentha arvensis*, due to water stress has also been reported by Misra and Shrivastava (2000). The higher value of crop yield obtained at 100% PWC might be due to more frequent application of water resulting in more adequate moisture in the active plant root zone and better utilization of nutrients. Under arid and stressed conditions, overall plant growth was reduced as a result of both biochemical disruptions and reduced cell enlargement, which in turn led to reduced leaf expansion and total leaf area and, therefore, reduced whole plant photosynthesis (Layer and Boyer, 1992; Mal and Lovett-Doust, 2005). That is the reason for low values of traits associated with growth and low yield in 30% PWC. At 30% PWC low crop yield obtained might be due to infrequent application of water resulting in a lack of moisture in the active crop root zone. Inadequate moisture may cause an insufficient amount of available nutrients for plants leading to low yield.

CONCLUSIONS

1. Water stress affected yield and growth of *Centella asiatica* L. Urban.

2. The highest growth traits like leaves number per ramet, petiole length, number of flower per ramet were obtained at 100% PWC.

3. The highest dry mass of plant was obtained for the treatment irrigated based on 100% PWC, while the least was obtained at 30% PWC.

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BIOLOGY AND MEDICINAL CHARACTERISTICS OF *CENTELLA ASIATICA* (L.) URBAN

Anjana Devkota and **P.K. Jha**
Central Department of Botany
Tribhuvan University, Kirtipur, Kathmandu
Email: devkotaa@hotmail.com; pkjhaprof@gmail.com

ABSTRACT

Centella asiatica is an important tropical medicinal plant.Although it is not in trade in Nepal but is widely used by pharmaceutical and ayurvedic companies.This plant is also used as a vegetable. Although a number of researches have been done to study its pharmaceutical and clinical importance, the life cycle and biology of this plant is poorly understood.This article briefly reviews the biology, ethnomedicinal uses, pharmacology and biochemistry of the plant.

Key words: *Centella asiatica*, clonal plant, triterpenoids, memory enhancer, ethnomedicine.

INTRODUCTION

Among the 33 species of *Centella* reported *Centella asiatica* (L.) Urban is considered the most important for its medicinal properties (Patra *et al.* 1998). It contains variety of ingredients but the active ingredients are asiaticoside (a triterpene glycoside) triterpenoid, brahmoside and brahminnoside (both saponin glycosides). The plant is listed in the Indian herbal pharmacopoeia, the German Homoeopathic Pharmacopoeia (GHP), the European Pharmacopoeia and the Pharmacopoeia of the People's Republic of China. Despite high demand in the herbal industry, there is no cultivation of this plant; the total supply of plant still comes from the wild. Negligible efforts have been made in developing proper agro-techniques of this plant. In India, due to large scale of unrestricted exploitation coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this species has markedly depleted and is now listed as threatened species by the World Conservation Union (IUCN) (Pandey *et al.* 1993) and an endangered species (Singh 1989, Sharma and Kumar1998). Contrary to this, there is no commercial collection of *Centella* in Nepal; neither there is recorded trade though it is found growing abundantly in tropical to subtropical zones of country up to an altitude 2200 m above sea level.

Nevertheless it is widely used in traditional medicine for treating various ailments like cough, fever, asthma, uric acid reduction, lowering high blood pressure, earache, etc. It is also used for memory enhancement as well as for longevity.

SPECIES DESCRIPTION

Scientific name: *Centella asiatica* (L.) Urban
Hydrocotyle asiatica L. (Syn.)
Family: Umbelliferae (Apiaceae)

Common name: Ghodtapre (Nepali), Kholchaghayan (Newari), Manduki (Sanskrit), Toprejhar (Gurung), Ghortapre (Tamang), Water pennywort, Indian pennywort (English).

Major documentation: National Register of Medicinal and Aromatic Plants: 76 (2004)
Plants and People of Nepal: 144 (2002)

Genetic Heritage of Medicinal and Aromatic Plants of Nepal Himalayas: 48 (2001); Ethnobotany of Nepal: 149 (2001); Ayurvedic Pharmacology: 64 (2001).

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British Museum (BM) West: PSW 5639. Cent.: SSW 2770. East: TI 6302545.

Catalogue of Nepalese Vascular Plants# 378.1:93 (1976); Medicinal Plants of Nepal: 43 (1970); Nepali Nighantu # 148:49 (1969); Chandra Nighantu #2:3-4.

Distribution and habitat

The plant is native to India, China, Nepal, Indonesia, Sri Lanka, Australia, Madagascar and Southern and Central Africa (Press *et al.* 2000). It is found throughout India and Nepal in moist places up to an altitude of 2200 m (tropical to subtropical region) and also on moist stone wall or other rocky sunny areas. It is an early coloniser of abandoned jhum (slash and burn agriculture) (Wankhar and Tripathi 1993). The plant can be grown in a variety of soils with moist, sandy or clayey loam, rich in humus. It is found associated with *Cyanodon dactylon, Bidens pilosa, Artemisia* sp., *Justicia* sp., *Alternanthera sessilis, Setaria* sp., *Ageratum conyzoides* and *Parthenium hysterophorus.*

Morphology

It is a small creeping herb. The stems are slender, creeping stolons, green to reddish green in color, interconnecting one plant to another with heart or kidney shaped leaves emerging alternately in clusters at the nodes. Leaves are slightly hairy and vary from 2-3 cm in length. Leaves have rounded apices with smooth texture and palmately netted veins. The leaves are variable in size; the petiole is usually 5 to 13 cm, sometimes longer than the lamina, which is 10 to 40 mm long and 20 to 40 mm, sometimes up to 70 mm, wide. The runners lie along the ground and leaves with their scalloped edges rise above on long reddish petioles. The rootstock consists of rhizome growing vertically down. They are creamish in color and covered with root hairs.

Inflorescence axillary simple umbel, peduncle 0.5-0.8 cm long, 2-5 flowered, ovate, membranous, and persistent. Flower minute, 0.3 cm in diam., hermaphrodite, actinomorphic, pink, calyx- teeth obsolete, petals 5, ovate, entire, imbricate, stamens 5, filaments short, anther bilobed, dorsifixed. Styles 2- from the base, filiformous, fruit about 0.3 cm long, laterally flattened, depressed ovate- globose, slightly pubescent when young, soon glabrate (HMG 1986).

REGENERATION

Reproduction

Plant reproduces by both vegetative and sexual means. In nature ramets play a greater role in population maintenance of *Centella asiatica* (Singh and Singh 2002). *C. asiatica* is a clonal perennial plant which flowers during April-August and seed setting commences around July-September and liberates after their maturation during summer i.e. June/July. Seeds are small, ca 2.75 mm length and 2 mm width. They are greenish brown to dark brown in color, weighs ca

1.75 mg/seed. The seeds of *Centella is* non dormant, germinate immediately after dispersal within one and a half month. The plant can show germination up to 74% in optimum laboratory conditions (A. Devkota, unpublished data). Seeds of *Centella asiatica* germinate during summer month of July-August when the temperature is above 25°C. It does not germinate in cold condition below 15°C.

Centella asiatica spreads by producing new plants on above-ground runners. The new plants can be separated from the parent plant once they have taken root. The plant perennates in the winter as creeping stem. Sprouting of perennated part takes place during growing season. The crop matures in three months and the whole plant, including the roots, is harvested manually.

***In vitro* regeneration**

In vitro propagation by tissue culture has been considered as an important tool for conservation and propagation of rare and threatened plants. A successful protocol for the regeneration of callus cultures of *Centella asiatica* was established by Patra *et al.* (1998) in which the stem and leaf explants were cultured on MS media supplemented with 2.0 mg/l kinetin and 4.0 mg/l of a-naphthaleneacetic acid (NAA). These were then regenerated after 4 weeks of subculture using 4.0 mg/l 6-benzyladenine (BA), 2.0 mg/l kinetin (Kn), and 0.25 mg/l NAA and 20 mg/l adenine sulphate. Full strength Murashige and Skoog (MS) medium (1962) has been used for most of the herbaceous species. In *Centella asiatica* multiple shoots were obtained from field-grown plants in MS medium supplemented with 1.0 mg/l BAP within 7 days of culture (Singh *et al.* 1999).

Banerjee *et al. (*1999) reported that initial sprouting in *Centella* required the presence of 6-benzyl aminopurine (BAP) (2 mg/l) and indole-3-butyric acid (IBA) (0.1 mg/l); however for multiple shoot induction a higher concentration of BAP (3.0 mg/l) and a lower concentration of NAA (0.05 mg/l) is required. Hossain *et al.* (2000) studied *in vitro* propagation of *Centella asiatica*, in which stem node explant of naturally grown plant was used for *in vitro* regeneration of multiple shoots. Various combinations of BAP and NAA in different concentrations were used in the regeneration of multiple shoots and a concentration of 1.0 mg/l BAP and 0.5 mg/l NAA was found superior in the optimum production of multiple shoots. Among the three auxins used in different concentrations, 0.2 mg/l IBA was found effective in the production of roots. Eighty per cent of the plantlets produced from *in vitro* culture method survived in the *ex vitro* condition. Measurement of chlorophyll a, chlorophyll b, carotenoid and soluble protein content of fresh leaf of both *in vitro* regenerated and natural plant represented non significant differences.

Tiwari *et al. (*2000) developed a protocol for rapid and large-scale *in vitro* clonal propagation of *Centella asiatica* by enhanced axillary bud proliferation in nodal segments isolated from mature plants. Subculturing of nodal segments harvested from the *in vitro* derived axenic shoots on the multiplication medium enabled continuous production of healthy shoots with similar frequency. MS medium supplemented with 6.7 –M BA and 2.88-M indole acetic acid (IAA) was found most suitable for shoot elongation. Rooting was highest (90%) on full-strength MS medium containing 2.46 -M IBA. Micropropagated plants established in garden soil were uniform and identical to the donor plant with respect to growth characteristics

Nath and Buragohain (2003) developed a method for rapid clonal propagation for *Centella asiatica* by shoot tip (2-3 cm long) culture. The shoot tips isolated from mature plants were inoculated on MS medium incorporated with BA alone or in combination with NAA and kinetin. The optimum number of shoots (3.38) with optimum number of leaves per shoot (4.25) was attained on MS medium supplemented with 4.0

mg/l BA and 0.1 mg/l NAA. On transferring the micro shoots on full strength MS medium supplemented with various concentrations of IBA (1.0-3.0 mg/l) and NAA (0.5-2.0 mg/l), profuse rooting (46.8 per shoot) was obtained in MS basal medium with 2.0 mg/l IBA with root length of 19.7 cm. Well rooted plantlets were acclimatized successfully by adjusting the temperature and humidity for 3-4 weeks after transfer to pots filled with sterilized vermiculite soil: sand (1:1) mixture.

Paramageetham *et al.* (2004) produced abundant somatic embryos from leaf segments excised from *Centella asiatica* when cultured on MS medium with 9.29 μM kinetin in combination with 2.26 μM 2-4-dichlorophenoxyacetic acid (2, 4-D). Granular, white, shiny clusters of callus developed after one week of culture, and then formed heart and cotyledonary stage embryos on the same medium after four weeks. Somatic embryos matured and germinated in the presence of MS medium containing 2.32 μM kinetin with (2.89μM) GA3. Plantlets were successfully transferred to pots containing a mixture of soil and vermiculite (1:1).

Sharma (2004) reported *in vitro* culture conditions of *Centella asiatica* for mass multiplication and callus culture and found that MS Basal + 3 mg/l BAP + 0.025 mg/l of IAA + 30 g/l sugar + 8.0 g/l agar gives the best result for culture initiation as well as in axillary shoot proliferation for number of leaves/shoot, number of shoots/node, number of nodes/explant and explant height. For rooting, MS + 0.5 mg/l IBA + 30 g/l of sugar + 8 g/l agar gave higher primary root /shoot with higher secondary roots. Green globular callus was best induced and proliferated in MS + 3 mg/l NAA + 1 mg/l kinetin + 30 g/l sugar + 8 g/l of agar.

Aziz *et al.* (2006) isolated protoplasts from cell suspensions initiated from leaf lamina and petioles using an enzyme mixture consisting of 1.5% (w/v) cellulase R10, 1.0% (w/v) macerozyme R10 and 0.5% (w/v) driselase in CPW salts solution with 13% (w/v) mannitol as osmotic stabilizer. Yields and viabilities of isolated protoplasts were 1.2 x 105 ± 0.1 g^{-1} fresh weight and 20.8 ± 4.4% for protoplasts from lamina-derived cell suspensions and 7.9 x 10^5 ± 1.5 g^{-1} fresh weight and 79.3 ± 13.4% for protoplasts from petiole-derived cell suspensions. Protoplasts from lamina explant-derived cell suspensions were cultured at plating densities of 0.25 x 10^5 – 2.0 x 10^5 protoplasts ml^{-1} in half-strength B5 based medium containing 0.1 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0.3 mg/l zeatin, dispensed as semi-solid agarose droplets (each approx. 70 µl in volume) in 5.5 cm diameter Petri dishes (10 droplets per dish). First mitotic divisions of protoplast-derived cells were observed after 4 days of culture at an optimum plating density of 0.5 x 10^5 protoplasts/ml, giving an initial plating efficiency at this time of 12.7±0.6%. After 42 days of culture, protoplast-derived cell colonies were creamy-white in color and each approximately 1 mm in diameter, with a final plating efficiency of 0.6 ± 0.2%. Cell colonies transferred to semi-solid proliferation medium containing 2, 4-D (4.0 mg/l) and zeatin (0.2 mg/l) were creamy-yellow in appearance, whereas colonies cultured on medium devoid of these growth regulators became light green and compact. In the case of protoplasts from petiole-derived cell suspensions, culture in Murashige and Skoog (1962)-based medium supplemented with 2.0 mg/l alpha-naphthalene acetic acid and 0.5 mg/l 6-benzylaminopurine resulted in an initial plating efficiency of 19.3 ± 4.2% at an optimum plating density of 1.0 x 10^5 protoplasts/ml.

Ethnomedicinal uses

Aerial part of *Centella asiatica* has wide range of ethnomedicinal uses (Table 1). The plant possesses antileprotic (Boiteau *et al.* 1949), antiulcerogenic (Cheng and Koo 2000), antifilarial (Chakraborty *et*

al. 1996), antibacterial (Srivastava *et al.* 1997), antifungal (Singh *et al.* 1999, 2000), antiviral (Cook and Shamman 1996) and wound healing (Tsurrmi *et al.* 1973) properties and is used as a tonic in Ayurvedic formulations. Clinical trials have also shown that it can help those with chronic venous insufficiency (Brinkaus *et al.* 2000). In Ayurveda, *Centella asiatica* is one of the spiritual herbs for improved meditation (Castiglioni 1958). Ethnomedicinal uses of this plant have been covered in most of the books on ethnobotany and medicinal plants of Nepal (Baral and Kurmi 2006, Joshi and Joshi 2001, Manandhar 2002, Shrestha and Shrestha 2004, IUCN 2004). There are few references describing detail methods of uses of *Centella asiatica* in ethnomedicine. According to Mahato and Chaudhary (2005), about 4 teaspoonfuls of leaf juice (juice obtained by squeezing 50 leaves between palms of hands) is taken orally in the morning for 2-3 weeks for its alleged cooling property to body and stomach. According to Shrestha and Dhillion (2003), plant parts are crushed with water, and juice is drunk against fever and throat ache, while fresh leaves chewed raw and swallowed for memory enhancement and blood purification in Dolakha district of Nepal.

The dried plant of *Centella asiatica* is powdered and this powder, mixed with hot water, is taken for gastric trouble; the fried plants are used in the diet of children for improving their memory (Ganesan *et al.* 2007). About 15 ml of leaf juice mixed with about 5 g mixture of 'Alainchee', Pipla', 'Jeethimadhu' and 'Gund' is given 2-3 times a day to cure cough. Leaf paste prepared with cow's urine is applied on nose and forehead to cure sinusitis 'Pinas' (Panthi and Chaudhary 2006).

Precautions and Safety: *Centella asiatica* has no known toxicity in recommended doses. The fresh plant may have a low potential for skin irritation, contact dermatitis has been reported. Orally consuming an excessive amount of *Centella asiatica* (i.e. overdose) can cause headache and transient unconsciousness. Also, chronic treatment may prevent women from becoming pregnant.

Table 1. Ethnomedicinal uses of *Centella asiatica.*

SN	Disorders/Uses	References
1.	Anorexia	Joshi and Joshi (2000), Manandhar (1990 b, 2002)
2.	Diarrhoea, Dysentry	Yadav and Mandal (2006), Gurung (2003), Shakya (2000)
3.	Blood purifier	Rai (2006), Shrestha and Dhillion (2003)
4.	Leprosy, Diuretic	Rai (2006), Rai *et al.* (2004), Lacoul and Lacoul (2000)
5.	Cuts and wound	Gurung (2003), Manandhar (1990 b, 2002), Shakya (2000)
6.	Headache	Shrestha *et al.* (2004)
7.	Tonic, Stomachic, Memory enhancer	Rai *et al.* (2004) Shrestha and Dillion (2003), Manandhar (1990 b, 2002), Sajem.and Gosai (2006), Lacoul and Lacoul (2000)
8.	Cure heating and tenderness of limb skin.	Rai (2004)
9.	Heat stroke, Urinary problem	Oli *et al.* (2005)
10.	Throat ache, Kidney stone, Fever, Genito -urinary problems, ENT problems	Shrestha and Dillion (2003), Sharma and Joshi (2000)
11.	Gastritis	Joshi and Joshi (2000), Manandhar (1990 b, 2002)
12	Body pain	Shrestha *et al.* (2004)
13.	Jaundice	Joshi and Joshi (2000), Manandhar 1990b,2002)
14.	Cooling property of body and stomach	Mahato and Chaudhary (2005)
15.	Depression treatment	Mamedov (2005)
16.	Conjunctivitis and other eye injury; stomachic, flatulence	Sajem and Gosai (2006)
17.	Pneumonia, skin diseases, toothache, indigestion.	Kunwar and Adhikari (2005), Sharma and Joshi, (2000), Sajem and Gosai (2006)
18	Piles, Brain tonic	Sharma and Joshi, (2000)
19.	Gas trouble, Memory enhancement	Ganesean *et al.* (2007)
20	Analgesic	Shakya (2000)
21.	Cough, Sinusitis	Panthi and Chaudhary (2006)

During prolonged treatment, especially with higher doses, the metabolism of asiaticoside to asiatic acid slows down proportionally to the plasmas asiatic acid content. This pharmacokinetic phenomenon should be considered for effective and safe treatment (Grimaldi *et al.* 1990).

Centella asiatica products

Centella asiatica is used for many herbal products. The common products that use *Centella asiatica* have been mentioned in Table 2.

Table 2. **Products from *Centella asiatica* used for various purposes** (Source: http://www.dermaxime.com/centella-asiatica.htm, accessed on Feb. 25, 2008)

• Healing Cream	• After Shave Balm
• Day Cream	• Night Cream
• Stretch Mark Gel	• Hand and Body Lotion
• Herbal Cellulite Gel	• Eye Gel
• Herbal Mud Face Mask	• Herbal Muscle and Joint Balm
• Herbal Face Bar	• Herbal Male Sexual Supplement

Culinary Uses

Centella is used as a leafy green in Sri Lankan cuisine. It is most often prepared as *mallung*; a traditional accompaniment to rice and curry, and goes especially well with vegetarian dishes such as *parippu* (dhal), and jackfruit or pumpkin curry. It is considered quite nutritious. In addition to finely chopped *Centella*, *mallung* almost always contains grated coconut and may also contain chillies, lime (or lemon) juice, dried fish, curry leaves, and spices such as fried mustard seeds.

Centella leaves are also used in the sweet "pennywort drink".

PHYTOCHEMICAL COMPOSITION

Due to various uses in traditional medicine of several Asiatic countries (Table 1) the aerial parts of *Centella asiatica* have been studied for its phytochemical composition. More than 70 constituents, belonging to triterpenoid saponins, polyacetylenes, flavones, sterols and lipids have been isolated from different parts of *Centella asiatica*. The major active constituents are triterpenoid saponins which include asiaticoside, madecassoside and madasiatic acid (Kartnig 1988). These saponins may prevent excessive scar formation by inhibiting the production of collagen (the material that makes up connective tissue) at the wound site. These constituents are also associated with promoting wound healing (Shukla *et al.* 1999a) as well as other biological activities. List of some important chemical constituents isolated from this plant are presented in Table 3.

BIOLOGICAL ACTIVITIES

Scientific basis of medicinal uses of ethnomedicinal plants can be assessed by evaluating the biological activities of their phytoconstituents. A few biological activities attributed to *Centella asiatica* are presented in Table 4.

TRADE AND CULTIVATION

As it is naturally available in Nepal, there is no practice of cultivating *Centella.* It can be transplanted easily through seeds or nodes containing root. It grows well in short span of time in appropriate wetland. According to report prepared by Export and Import Bank of India, *Centella asiatica* is one of the important medicinal plants in the international market (Rao 2000). Since there is a high demand for *Centella asiatica* in India (135 t, CERPA 2004/2005), there is a

good opportunity for its cultivation at commercial scale. It would be financially beneficial to cultivate this plant and bring it under trade in Nepal. If we are able to bring it in trade that may be supportive for raising economy of rural community. However, agrotechnology for cultivation of *Centella asiatica* is not well developed. Methods have been developed for mass production of plantlets by tissue culture (Tiwari *et al.* 2000, Sharma 2004).

Table 3. Important chemical constituents present in *Centella asiatica.*

SN	Chemical constituents	Plant parts	References
1.	Asiatic acid	Aerial parts	Yoshida *et al.* (2005), Srivastava *et al.* (1997)
2	Asiaticoside A	Aerial parts	Srivastava *et al.* (1997)
3.	Asiaticoside C, Asiaticoside D, Asiaticoside E, Asiaticoside F	Whole plant	Jiang *et al.* (2005)
4.	3-glucosylquercetin, 3-glucosylkaepferol and 7-glucosylkaempferol	Leaves	Srivastava *et al.* (1997)
5.	Galactose, Galacturonic acid, Pectin Raffinose, Rhamnose, Riboflavin, Thiamine, Xylose	Aerial parts	Wang *et al.* (2005)
6.	Brahmic- Acid, Brahmoside, Betulic acid Brahminoside, Isobrahmic acid, Centoic acid, Centellic acid, Centelloside, Indocentoic acid, Glycine, Aspartic acid, Glutamic acid, Isothankuniside, Thankunic acid, Isothankunic-acid,Alanine and Phenylalanine, Stigmasterol, Stigma sterol-β-D-glucopyranoside, Hyperin, Hydrocotyline, Germacrene –D, Kaempferol	Whole plant	Srivastava *et al.* (1997)
7.	Asiaticoside B, Madasiatic acid, Madecassic acid, Medicassoside, Mesoinosital, Olean-13-ene triterpene Centella sapogenol A, Centella saponin A, Centella saponin B Centella saponin C, Centella saponin D, Sceffoleoside, Sceffoleoside A	Aerial parts	Matsuda *et al.* (2001)
8.	Promolic acid, Rosmarinic acid, Ursolic acid, 8- acetoxy-1, 9 pentadecadiene, 2α, 3α-dihydroxyurs-12 en-28-oic acid, 3-epimaslinic acid, Corsolic acid, Ursolic acid, 8- acetoxy-1, 9-pentadecadiene, 2α, 3α-dihydroxyurs-12 en-28-oic acid, 3-epimaslinic acid	Aerial parts	Yoshida *et al.* (2005)
9.	Scheffuroside	Whole plant	Jiang *et al.* (2005)
10.	Saponin	Whole plant	Matsuda *et al.* (2001), Wang *et al.* (2005), Yoshida *et al.* (2005)
11.	Vellarine, Elaidic –Acid, Lignoceric- acid, Beta carotene	Whole plant	http:/www.chromadex.com/Phytosearch/gotukola.htm, accesed on Nov. 2006

Table 4. Biological activities attributed to *Centella asiatica.*

Activity	References
Against Anaemia, Antihypertensive	Jayatilake and Macleod (1987)
Blood purifier, Diuretic	
Antidepressive	Sakina and Dandiya (1990)
Anti allergic and anticanceric	Kan (1986)
Anti-inflammatory	Suguna *et al*. (1996)
Antilipid peroxidative	Katare and Ganachari (2001)
Antiallergic, Antioxidant Antiviral, antiproliferative	Youdim and Joseph (2000)
Antithrombic	Cook and Samman (1996)
Immulomodulant	Plohmann *et al.* (1994)
Sedative	Sakina and Dandiya (1990)
Ulcer preventive	Huriez (1972)
Memory improvement	Veerendrakumar and Gupta (2002), Mukharji (1953)
Urethritis	Jagannath and Ng (1999)
Vasdilatory	Cook and Samman (1996)
Free radical scavenging	Jayashree *et al.* (2003)

CONCLUSION

There is still a wide scope for exploring different aspects of *Centella asiatica*. There are no established agro-techniques for promoting its cultivation. Considering the range of different niches occupied by the plant, there is a possibility that many ecotypes and/or chemotypes of *Centella asiatica* exist. It would be interesting to study the morphological, molecular and biochemical variations among different populations of *Centella asiatica.* There are of course no established varieties or lines of *C. asiatica*; hence a strong need is felt to screen the different chemotypes growing at different phyto-geographical locations. Similarly biodiversity studies at biological and genetic levels will enable the research community to realize the extent of variability within existing germplasm of *Centella asiatica* and help in conservation and promotion of the plant.

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USES AND SUSTAINABLE HARVESTING OF *CENTELLA ASIATICA* (L.) URBAN

Anjana Devkota and **Pramod Kumar Jha**
Central Department of Botany
Tribhuvan University, Kirtipur, Kathmandu, Nepal
Email: devkotaa@gmail.com, pkjhaprof@gmail.com

ABSTRACT

Centella asiatica is one of the widely used tropical medicinal herbs of Nepal. The plant has been used extensively as memory enhancer and brain tonic. This plant has been entirely supplied from wild for traditional as well as industrial uses. There has been no report of commercial cultivation of this plant. Rapid loss of *C. asiatica* has been taking place in the wild due to habitat destruction, over-exploitation and unsustainable collection. The best way to conserve this plant is by protecting its habitat as well as by its cultivation. The best condition required for cultivation of *C. asiatica* is moist, shady place with sandy loam soil. This article briefly reviews the uses and harvesting practice of the plant in Nepal.

Key words: Medicinal uses, memory enhancer, harvesting practice.

INTRODUCTION

Centella asiatica (L.) Urban (Fam. Apiaceae) is commonly known as 'Ghodtapre' in Nepal and 'Indian pennywort' or 'Mandookaparni' in India. The herb is known as 'Brahmi' in Unani medicine, 'Manukaparni' in Ayurveda and 'Gotukola' in western world. It is a slender, tender, faintly aromatic herb. The stems are slender, upto 2 m long, stolon creeping, green to reddish green in color, interconnecting one plant to another. It has long-stalked, green, reniform leaves, with rounded apices which have smooth texture with palmately netted veins, petiole 2-6 cm long and 1.5-5 cm wide, lamina circular to reniform, rather broader than long, more or less cupped, entire to crenate, margin dentate, and glabrous in both side. The flowers are pinkish to red in color, born in small, rounded bunches (umbels) near the surface of the soil. Each flower is partly enclosed in two green bracts. The hermaphrodite flowers are minute in size (less than 3 mm), with 5-6 corolla lobes per flower. Each flower bears five stamens and two styles. Fruit is 4 mm long, oval to globular in shape, hard with thickened pericarp, often crowned by the persistent petals (Kirtikar and Basu 1987).

The *Centella asiatica* reported to contain more than 70 chemical constituents (Devkota and Jha 2008a). The major constituents reported from this plant are triterpenic acids (Singh and Rastogi 1969, Yoshida *et al.* 2005), glycosides (Rastogi and Mehrotra 1993), and volatile and fatty oil (Chopra *et al.* 1956). Besides these, the plant also contains flavonoids (Zainol *et al.* 2003), oligosaccharides, centellose, quercetin (Matsuda *et*

al. 2001). The ash contains chloride, sulphate, phosphate, iron, calcium, magnesium, sodium and potassium (Malhotra *et al.* 1961).

Distribution and Habitat

Centella asiatica is native to the wetlands of Asia (China, India and Malaya), but currently this plant is found widely in South East India, Sri Lanka, parts of China, the Western South Sea Islands, Madgascar, East and South Africa, Turkey, and the southeastern United States (Press *et al.* 2000). It is found throughout India and Nepal in moist and open places up to an altitude of 2200 m (tropical to subtropical region) and also on moist stone wall or other rocky sunny areas. In Nepal it spreads widely from eastern to western region from tropical to sub tropical belts (Fig. 1). But the growth traits and yield of the plant is higher in shady and relatively undisturbed site (A. Devkota, unpublished data).The plant can be grown in a variety of moist soils from sandy, clayey loam to humic soil. In grasslands of central Nepal, the species is found associated with *Cyanodon dactylon*, *Bidens pilosa*, *Trifolium repens* (Devkota and Jha 2008b).

Fig. 1. Distribution of *Centella asiatica* population in Nepal.

Medicinal Uses

In Nepal, this plant is one of the most commonly used medicinal herbs for a wide range of disorders (Devkota and Jha 2008a). The plant is cooling, alternative, cardiotonic, nervine tonic, sedative to nerves, tonic to vital organs (liver, kidney, brain). It is used in disease of skin, nerves and blood and taken as tonic for accelerating nervous activity and for improving youth, longevity and memory. The leaves are said to be useful in treatment of ulcerations, psoriasis, leprosy, tuberculosis, cardiac problem, asthma, bronchitis, abdominal disorder, headache, fever, and for would healing (Reviewed by Devkota and Jha 2008a). Recently, the plant extract has been used as constituents of "anti-ageing" skin creams (Jamil *et al.* 2007).

In Chinese medicine the herb is used for dysentery and summer diarrhea, vomiting, jaundice urinary calculi, epistaxis and scabies (Anonymous 2000). In Homoeopathic medicine it is used for skin disease associated with itching and swelling. It is used in inflammation and ulceration of uterus, eczema, elephantiasis, ascariasis and in granular cervicites (Anonymous 2000, Singh and Rastogi 1969).

Cosmetic uses

Centella asiatica has also been used widely in cosmetics. It has the properties of formation of lipids and proteins for healthy skin, anti-cellulites, skin tightening, and skin regenerative. It has been used as anti- wrinkle for eyes and facial skin, for the treatment of acne induced blemishes, rapid renewal of supporting fiber network and to boost immuno-depressed skin (Michael 2003).

Other Uses

Along with the use of *Centella asiatica* in medicine, the plant is also taken as a vegetable as its leaves are particularly rich in carotenoids, vitamin B and C (Ng 1998). The leaves of *C. asiatica* are used as vegetable (in curries and salads) in Kerala, India. In Malaysia, it is cooked as vegetables or eaten raw as salads and with rice.

It is also commonly used in porridge for feeding pre-school children in Sri-Lanka for combating nutritional deficiencies (Zainol *et al.*2003). Meanwhile, in the Hawai Islands, the leaves are fed to cows to increase milk yield and also to poultry and rabbits.

Threats

Centella asiatica prefers open grassland. Due to urbanization, habitat destruction and growing number of invasive species like *Parthenium hysterophorus*, the population of *C. asiatica* is being threatened (Karki 2009, personnel observation of AD).The plant is commercially threatened due to its high medicinal value and multipurpose uses, spontaneous collection and over exploitation by local people. Though plant flourishes well during summer season, local people collect this plant throughout the year. The untimely harvesting practice may also threaten the plant in wild.

The plant has been listed as source of drug in the Indian herbal pharmacopoeia, the German Homoeopathic Pharmacopoeia (GHP), the European Pharmacopoeia and the pharmacopoeia of the People's Republic of China. Despite a decent hold in the herbal industry, the plant is still collected from the wild (not cultivated) and negligible efforts have been made in developing proper agro-techniques for cultivation of this plant. Because of large scale unrestricted exploitation of this natural resource, coupled with no cultivation and insufficient attempts for its replenishment, the wild stock of this plant species has markedly depleted. It is listed as threatened species by the International Union for Conservation of Nature and Natural resources (IUCN) (Pandey *et al.*1993) and as an endangered species in India (Singh 1989; Sharma and Kumar 1998). According to the report prepared by the Export and Import Bank of India, *Centella asiatica* is one of the important medicinal plants in the International market of medicinal plant (Rao 2000).

Sustainable Harvesting Practice

Centella asiatica propagates from seeds and stolon. A potential sustainable harvesting technique could be to harvest leaves from densely populated site for medicinal purposes instead of uprooting the whole plant as is the common practice. This would ensure plant regeneration. For sustainable regeneration of plant, it is better to harvest plant during actively growing season i.e. during summer. The best way to conserve this plant is by protecting its habitat as well as by its cultivation. Unfortunately, there has been no significant attempt for its cultivation.

In Nepal the plant is widely used by locals for traditional medicine and in pharmaceutical companies. All required amount is supplied from the wild. As it is naturally available in Nepal, there is no practice of cultivating *Centella asiatica.* It can be transplanted easily through seeds or nodes containing root. It grows well in short span of time in appropriate wetland. Though the plant occurs in diverse habitat, the best suitable condition for cultivation of *C. asiatica* is moist (90-100% field water capacity) (Devkota and Jha, unpublished data) and partially shaded (i.e. 30% shade) habitats (Devkota and Jha 2010). This plant exhibits better growth in sandy loam soil than in clay and pure sand (Devkota and Jha 2009).

CONCLUSION

Centella asiatica is one of the widely used medicinal herbs in Nepal. Rapid loss of *C. asiatica* is taking place in the wild due to habitat destruction, over-exploitation and unsustainable collection. Invasive plant like *Parthenium hysterophorus* is also causing loss of population of this plant. Therefore, there is a need for developing proper agro-technology for enabling cultivation of this herb. There is also a need for spreading awareness about sustainable collection of this plant. *C. asiatica* can be cultivated in moist, shady places with sandy loam soil.

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