DISCUSSION

Exploitation of plant kingdom has led to threatening global biodiversity. This can result in extinction at the population level and even extinction of whole species. Therefore conservation of biodiversity necessitates choices among areas, taxa and land use patterns (Lynch, 1999).

Overexploitation of indigenous plant for medicinal purpose has social and ecological impact, on our biodiversity. From social perspective, people tend to lose some indigenous plants for medicine harvest, due to overexploitation. In this case, some diseases are no longer cured because of the shortage of medicinal plants. In ecological perspective, plants are green glue that bind soil and keep animals alive in the biodiversity. *Lasia spinosa* is a traditionally important plant in indigenous system. The tender leaves and rhizomes are used as vegetable and are recommended for a variety of diseases in Ayurveda medicine. Rhizomes are rich source of soluble and insoluble dietary fibers. Dietary fibers in the diet play an important role in the physiology of gastrointestinal tract especially in the patients with hyper-cholesterolaemia and type 2 diabetes mellitus (Wikramanayake, 1996). Moreover, there is enormous number of reports about that *Lasia spinosa* has been used in traditional medicines globally. Much of the world's population depends on traditional medicine to meet daily health requirements. About 80% of people in the developing countries still practice traditional medicine based health care remedy till date (Verma and Singh, 2008). Use of plant-based remedies is also widespread in many industrialized countries and numerous pharmaceuticals are based on or derived from plant components (Anis et al., 2012; Krishnaraju et al., 2005).

*Lasia spinosa* is cosmopolitan in North Eastern region. It is commonly used by the local people as pot vegetable and is given to lactating mothers. In order to boost their nutrition levels, most rural dwellers in many parts of Kokrajhar district, especially the Bodo communities have resorted to the administration of the leaf extract of *Lasia spinosa* as the cheapest source of multivitamins (Shefana and Ekanayake, 2009). The nutritional and medicinal value of these plants lies in some chemical substances that have a definite physiological action on human body. The
most important bioactive constituents of this plant are alkaloids, terpenoids, carbohydrates and protein compounds. It is evidently witnessed that the plant is rich in carbohydrates such as 35.74% in dried extraction. No major threats have been reported and the species is therefore listed as ‘Least Concern’ version - 3.1 according to IUCN red data list.

5.1. Morphological and anatomical features

Lasia spinosa was known to be only species of the genus Lasia until 1997 when a wild population of Lasia concinna was discovered in a farmer’s paddy field in West Kalimantan, Indonesia. There is no report of availability of L. concinna in India till date. Plants exhibit natural variations in their form and structures. This variation is most easily seen in the leaves of a plant, though other organs such as stems and flowers may also show variation. There are three primary causes of variations: positional effects, environmental effects, and juvenility. Lasia spinosa was reported to possesses three morphological forms based on the leaf character viz. i) sagittate, ii) lamina dissected and iii) a mixed of sagittate and lamina dissected among them only two were available in Assam. Earlier findings by Sultana et al., (2000) had marked the morphological forms of L. spinosa only on the basis of leaf character. In this present study, the morphological forms were identified on the basis of leaf character and inflorescence colour into two morphological forms, which are available in Assam viz., mixed form (Morphotype - I) and lamina-dissected form (Morphotype - II). The petiole of morphotype - I was relatively longer than morphotype - II. There is a variation in the flower colour, lobe size, peduncle size etc. in both the morphotypes.

According to World Health Organization (WHO) the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any analysis are undertaken.

Anatomical study may provide useful information for establishing interrelations between taxa at the species and supra-species levels. In the present study anatomical features on both the morphotypes were noted. The location and activity of plant roots play important ecological roles, affecting processes such as
competition for water and nutrients, direct plant-plant interactions, dynamics of mycorrhizal fungi and rhizosphere bacteria, and biogeochemistry of soils (de Kroon et al., 2003; Reynolds et al., 2003; Schenk, 2006) and may therefore strongly influence ecosystem responses to global change.

Anatomy of root in both the morphotypes revealed that anatomical features were more or less same except the variation in arrangement pattern on vascular bundles. A slight variation on petiolar anatomy and leaf architecture was found on both the morphotypes.

5.2. Karyotypic analyses

In the present investigation, karyomorphological as well as cytological studies were undertaken on two morphotypes of Lasia spinosa aiming to understand variation between the two morphotypes in chromosome number and in detail organization, despite their construction is from the same macromolecules. The idea of karyotype as a characteristic especially and exclusively applied to genetic definitions or to any other systematic unit is not tenable. Karyotypes are useful as they permit rapid recognition of any observation either in chromosome number or morphology or both. Karyomorphological studies traces the phylogenetic relationships among the flowering plants (Iwatsubo and Naruhashi, 1991). It helps to ascertain the pattern of genetic diversity in plants which may be exploited for further breeding for increased plant productivity required for the fulfillment of basic needs of mankind. Araceae shows a wide range of basic chromosome numbers from 8 to 22. The commonest basic number in the family is \( x = 14 \). Even though the haploid number 7 has not so far been found in any aroid, \( x = 7 \) is assumed to be the original basic number of the family and that \( x = 14 \) is derived by polyploidy (Mookerjea, 1955; Jones, 1957).

Karyotype analysis provides the species to correct taxonomic status. It is the basic tool to provide taxonomic character. It has been reported that species often show similarity in gross chromosome morphology but they differ from each other in small details in chromosome morphology like the centromere, secondary constriction, number and size of satellites and variation in total chromatin length (Sharma and Mallick, 1965; Mazumdar and Riley, 1967, 1972; Riley et al., 1968; Zarco, 1986;
Borah, 1998). In the present study, marked differences were found in the karyotypes of 2 forms of *Lasia spinosa*. These were:

(i) the 2n chromosome number of both the form was different
(ii) presence of distinct centromeric formulae
(iii) different length in 2n chromosome complement
(iv) variation in volume of chromosomes and
(v) variation in relative length of chromosome.

Karyotypic analysis of *L. spinosa* (Morphotype - I) reveals that it has diploid no. of chromosome 2n = 26 and centromeric formula is 7M + 14m + 5sm = 26, which is not in agreement with the earlier report where it was found as 14m + 11sm + 1t = 26 (Sultana *et al.*, 2000). According to earlier report there are 5 satellite chromosomes in mixed form but in our report satellite chromosome was not observed under microscopic observation. Further, it was reported that the mixed form is intermediate between metacentric and sub-metacentric chromosome but here it was found the presence of median chromosomes.

The chromosome number in *L. spinosa* (Morphotype - II) was found to be diploid 2n = 24 unlike the earlier findings where the number was 2n = 26. These changes in the chromosome number and structure may be due to the involvement of chromosome re-patterning coupled with addition or deletion of certain specific chromosomal segments (Lavania and Sharma, 1983; Cox *et al.*, 1998). Centromeric formula found in this study was 4M + 14m + 3sm + 1st + 2t = 24 varies from earlier findings 9m + 15sm + 2st = 26 in the lamina dissected form (Sultana *et al.*, 2000) In the earlier studies two sat-chromosome was recorded but no sat-chromosome was observed in this study.

The 2 forms had median, metacentric and sub-metacentric chromosomes in their karyotypes. In addition to above, morphotype - II had sub-telocentric and telocentric chromosomes in their karyotypes.
In morphotype - I, the chromosome length varied from 2.31 µm - 4.08 µm while their volumes ranged from 0.034 µm³ - 0.370 µm³. The total genomic chromosome length was found to be 84.38 µm which is also contrast to earlier report where it was mentioned as 74.46 mm (Sultana et al., 2000). In morphotype - II, the chromosome length varied from 1.09 µm to 4.42 µm while their volumes ranged from 0.015 µm³ to 0.401 µm³. The total genomic chromosome length was found to be 67.83 µm which is contrast to earlier report of 57.78 mm. However, relative chromosome length, total genomic volume of the somatic chromosome as well as TF% was not reported by earlier workers.

In conclusion, this data support the idea that morphotype of *L. spinosa* studied here is intermediate between primitive and advance character because it possesses median, metacentric, sub-metacentric, sub-telocentric and telocentric chromosomes. The variations might have been brought about due to tandem or structural alterations in chromosomal contraction at metaphase resulting into variation in chromosome size (Lavania, 1985). Such type of karyotypic variation in the two morphological forms may also occur because of differences in the repetitive DNA sequences which eventually make up the genome size and different chromatin and heterochromatin densities along the chromosome arms (Quicke, 1997; Schmidt et al., 1998). Karyotypic analysis through chromosome characterization could be exploited to trace the evolutionary relationships among plant species (Che et al., 1984). These karyomorphological reports will provide a new insight for further characterization of this important plant in molecular level.

Our data bring to light that the two form of *Lasia spinosa* collected from Assam, India vary in their chromosome number and both the form has symmetric karyotype. The variation in the two findings might be due to the habitat distribution and climatic effect.
5.3. *In-vitro* propagation

Tissue culture, maintenance and propagation of plant parts in axenic culture under controlled environmental conditions are quite important (Murthy *et al.*, 2008). It facilitates mass propagation and preservation of valuable medicinal plant. *In vitro* plant propagation system serves as an alternative approach for the production of bioactive compounds from medicinal plants. To ensure reproducible quality of herbal products, proper control of starting material is utmost essential. Thus in recent years there has been an emphasis in standardization of medicinal plants for conservation of genetic stock of threatened species to secondary metabolite production in important plant taxa and year round supply of disease free quality planting material for commercial cultivation. The results obtained in our experiment suggested that *in vitro* plantlet regeneration using micro cuttings of rhizome containing bud may be used for direct clonal propagation and conservation with a low risk of generating disease free quality planting material in large scale for *Lasia spinosa*.

In this study, an *in-vitro* propagation protocol has been developed for *L. spinosa* using microcuttings of rhizome bud. The plants showed direct organogenesis from the microcuttings of rhizome bud which was found to be more suitable for regeneration when cultured on MS medium using various concentrations of BAP (1.0-4.0) and kinetin (1.0 - 4.0) separately or in combination with low concentration (0.5 and 1.0 mgL⁻¹) of auxin (NAA). It was observed that BAP in combination with NAA was more effective for shoot induction. Stanly *et al.*, (2010) initiated *in vitro* shoot cultures from the rhizomatous buds on MS basal medium. The best conditions for propagating *Homalomena pineodora* was found to be on MS medium supplemented with 3% sucrose and 0.5 mg L⁻¹ BA (6 - benzyladenine) under 24 hrs of cool fluorescent light which produced an average of 3.8 shoot per explant.

Treatments of BAP and NAA, 3.0 mg/L BAP + 1.0 mg/L NAA was found to be suitable and showed better response in *L. spinosa*. In this concentration, 90.6% explants induced to develop shoots. The number of shoot as well as length of shoot per explant was recorded as 3.6 ± 0.55 and 3.42 ± 0.10 cm respectively. BAP is considered one of the most suitable cytokinin for the multiplication of axillary buds reported by many authors (Sharma *et al.*, 1995; Martin *et al.*, 2002; Joshi and Dhar,
In the present investigation, combination of BAP with NAA was found more suitable than BAP and kinetin alone. But, highest shoot length was observed in low concentration of BAP \( i.e., \ 4.21 \pm 0.06 \text{ cm} \).

Lamina explants exhibited more potential for callus formation when they contained midrib (visual observation), which was agreement with results reported by Kumar \textit{et al.}, (1992); Bejoy \textit{et al.}, (2008). Callus induction was observed from leaf explants. Highest percentage of callus induction (94.1 \( \pm \) 2.8) in \textit{L. spinosa} was observed from leaf explants on MS medium supplemented with 3.0 mg/L of BAP and 1.0 mg/L of NAA in combination. \textit{Anthurium anderanum} from family Araceae, is a plant with a high commercial value. Callus with the highest fresh and dry weight was produced on modified MS medium from leaf explant containing 0.1 mg/L 2, 4-dichlorophenoxy acetic acid (2,4-D) and 1.5 mg/L 6-benzylaminopurine (BAP) (Farsi \textit{et al.}, 2012).

Rhizomatous bud of \textit{L. spinosa} exhibited multiple shoot proliferation on MS medium supplemented with BAP + NAA. The quality of shoots and the overall growth response in terms of average shoot length was better in this growth regulator combination. A comparatively lower response was recorded when BAP or kinetin was added singly in the medium. Review of literature indicates that the addition of PGRs (IAA or NAA) in the culture medium improved the response in a number of species in terms of shoot growth. Raomai \textit{et al.}, (2013) performed culture on MS medium through rhizome bud explants supplemented with various concentrations of cytokinins to induce multiple shoot formation for micropropagation. The highest number of shoots was achieved in MS medium supplemented with 2.0 mg/L 6-benzylaminopurine. The regenerated shoots rooted most efficiently on half-strength MS medium supplemented with 0.5 mg/L \( \alpha \)-naphthalene acetic acid (Asthana \textit{et al.}, 2011).

Two different media were used for developing the tissue culture technique of \textit{L. spinosa}., MS (Murashige and Skoog, 1962), and Gamborg B\textsubscript{5} (GB\textsubscript{5}), MS was the most suitable medium for this particular plant. The MS medium supplemented with auxin or cytokinin alone or in combinations induced highest percentage of shoot
proliferation and maximum number of shoots from the shoot tip of *Musa* sp var. “Yangambi”. The shoot tips were cultured on MS media supplemented with different concentrations of BAP (0, 2, 4, 6 and 8 mg/l) with or without IAA at concentration of 0.34 mg/L (Ngomuo et al., 2013). There is a synergistic requirement of both auxin and cytokinin to induce cell division and growth in plant tissue culture. The use of cytokinin in plant nutrient media for *in vitro* culture depends on plant tissue growth stage and expected end product. Experiment on whole plant and excised tissues have largely establish the existence of antagonistic and additive interactions linking these two types of plant growth regulators (Kothari et al., 2010). The results of the present study were more or less coincided with previous observations (Gubis et al., 2003; Mariani et al., 2011; Raomai et al., 2013). In the present study we optimized a protocol for large scale multiplication of *L. spinosa* using rhizomatous bud as explants.

*In vitro* formed shoots were excised and rooted on a separate root inducing half strength basal MS medium. Regenerated shoots thus formed were carefully excised and then rooted on basal MS medium.

Rooting on proliferated shoots of *Anthurium andreanum* were successfully obtained on addition of PGRs viz., IBA (0.0, 0.5, 1.0 and 2.0 mg/L), NAA (0.0, 0.05, 0.1 and 0.25 mg/L) and KIN (0.0 and 0.2 mg/L) (Raad et al., 2012). In the present study auxins viz., NAA and IBA were used singly to induce rooting from *in vitro* raised shoot lets. The maximum results on rooting were obtained on half strength supplemented with IBA (0.5 mg/L) then NAA. Mariani et al., (2011) performed rooting of plantlets of *Aglaonema* on MS medium containing 3 mg/L indole-3-butyric acid (IBA) Rooting is usually induced by auxins, and IBA is more effective for rooting compared with other auxins as reported for *Anthuriums* (Malhotra et al., 1998; Puchooa and Sookun, 2003; Jahan et al., 2009). Martin et al., (2003) cultured shoots of *Anthurium andreanum* in medium supplemented with 0.54 μM NAA and 0.93 μM kinetin for *in vitro* rooting.

*Ex-vitro* adaptation of a micropropagated plant to a greenhouse or a field setting is indispensable as there is, in general, a noteworthy discrepancy between the auxenic *in vitro* environment and the greenhouse or field condition. Well rooted plants of present investigation were transferred to plastic cups containing soilrite for
hardening and kept under controlled condition. The plants were transplanted to vermiculite medium; they soon regenerated fresh shoots and roots after one week. Later they were transferred to the field and the survival rate was 70%. The competent micropropagation technique described in the study may be highly beneficial for raising quality planting of medicinal plant for commercial and off season cultivation which is not only help the ex-situ conservation but also helpful in the restoration of genetic stock of the species. Mahanta and Paswan, (2001) successfully transferred in vitro Anthurium plantlets in the plastic pots containing the growing medium of soilrite-perlite at the ratio of 10:1 and reported 60% survival rate after four weeks of transfer. Effect of vermicompost and sand mixture (1:3 v/v) in ex vitro establishment under greenhouse followed by net house condition was reported by Martin et al., (2003), where 95% survival rate was achieved. Maximum survival rate of 98% was reported by Han and Goo, (2003) in cultivar Atlanta using a combination of vermiculite and perlite (1:1 v/v) as growth substrate.

In a report by World Health Organization, it was acclaimed that a high percentage of the world’s population are using herbal medicine as drug and there is a growing interest in the use of traditional medicines (Tilburt and Kaptchuk, 2008). However, herbal medicines, like other natural resources, have very limited sources. Thus, artificial regeneration of herbal plants becomes important. Plant tissue culture system offers a tool for a large scale production of genetically similar plants (Islam et al., 1993; Wawrosch et al., 1999). The traditional propagation of hybrid is challenging due to the growth of a low number of new plants from the base (Martin et al., 2003). Therefore, plant regeneration in vitro is an alluring alternative for mass multiplication of outstanding cultivars at faster rates than conventional methods.

Our findings have paved a way for future investigations on the other modes of regeneration (e.g., haploid production, anther culture and protoplast culture etc.) and use of advanced biochemical and molecular markers to assess the differential stages will help to unknot novel mechanisms for the development of more proficient strategies with improved in vitro methods.
5.4. ISSR marker based phylogenetic analyses

Molecular marker based phylogenetic analysis have already proved valuable in molecular plant systematics, especially in studies on genetic diversity and gene mapping. Among the different methods so far available, ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of plant species including crop plants (Reddy et al., 2000). Superiority of ISSR-PCR over other marker techniques has been brought out in such investigations by various workers. ISSRs were more useful for the analysis of diversity in the different genus plants in terms of quality and quantity of data output as compared to RFLP and RAPD (Salimath et al., 1995). Significantly, the efficiency of the technique was evident in characterization even at the varietal level of a species by different workers. For instance, three 5’ anchored primers together could distinguish 20 cultivars of Brassica napus (Charters et al., 1996). ISSR is the marker of choice for assessment of genetic diversity in cocoa (Charters and Wilkinson, 2000), gymnosperms (Tsumura et al., 1996) and even fungi (Hantula et al., 1996). In a study on white lupin, it has been demonstrated that among 10 primers used any two were sufficient to distinguish all the 37 accessions studied (Gilbert et al., 1999). The use of such highly informative primers lowers the cost, time and labor for diversity analysis.

In this study, a panel of 8 ISSR primers has been used to assess the genetic diversity between the two morphotypes of Lasia spinosa available in Assam, India. Twenty four of the 56 bands were shared by these two morphotypes, indicating a common evolutionary history or homoplasy as because these two morphotypes belong to the same species. While the remaining 32 bands reflect divergence between the morphotypes. If one morphotype was derived from the other, producing a progenitor–derivative pair and this was associated with a reduction in effective population size that would expect to observe only a subset of alleles in the derived morphotype (Gemmill et al., 1998). About 57 % of polymorphisms (32 of 56 bands) that are specific to each other between the two morphotypes of Lasia spinosa strongly suggest that though these two morphotypes belong to the same
species, but are genetically 57% different from each other forming two morphological variations.

With this study it can conclude that the analyses of ISSR markers was useful for study the genetic relationships between the two morphotypes of *Lasia spinosa*, providing the ISSR markers a powerful tool for the generation of potential fingerprinting diagnostic markers for these two morphotypes of *Lasia spinosa*. Also the phylogenetic analysis on the basis of ISSR-derived phenogram would be valuable information for suitably selection of these important groups of rare and endemic medicinal plants to exploit for bioprospection.