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A review on influence of floral biology, pollination efficiency and conservation strategies of endangered medicinal plant, *Rauvolfia serpentina* (L.) Benth. *ex* Kurz

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Abstract

Utilization of medicinal plants are being currently increasing drastically in the entire world due to its increasing demand of herbal medicines, natural health products and secondary metabolites of medicinal plants.The current manuscript is mainly emphasized on information based on its botanical description, taxonomical classification, study on floral biology and its pollination efficiency, its global status of conservation, conservation strategies along with methods of propagation including *in vitro* and *ex vitro* have been collected and compiled from abundant numbers of scientific literatures. Because of magnificent demand of medicinal properties of *Rauvolfia serpentina* (L.) Benth. *ex* Kurz, it has become highly essential to study in depth about floral biology which would further prove to be resourceful for hybridization programme and after all, information of floral biology is a prerequisite in assessing overall reproductive potential of the species. Relatively, pollination is also one of the most important mechanisms confined to preservation and conservation of biodiversity in broad-spectrum life of earth. Since, psychophily mode of pollination is prevalent in *R. serpentina* due to its tubular flower structure, and hence conservation of these pollinators is a necessity for preventing threatened species from further extinction. In recent years, products of *R. serpentina* particularly manufactured from roots are adulterated with wild plants since suppliers from wild sources are limited, hence due to an increase in rate of exploitation. It has hindered in continuation of constant and steady supply. Consequently protection of forest based genetic resources has turned out to be a global concern which urges expansion of effective conservation strategies at local, national and international level. *R. serpentina* can be propagated by several methods involving seeds, root cuttings, root stumps and stem cuttings. However, conventional propagation methods practised in this species seems to face some major constraints indicating poor seed viability and germination. Under these conditions, application of *in vitro* conservation methods such as *in vitro* culture and micropropagation along with synthetic seed production and techniques of cryopreservation involving hairy root culture enhanced in liquid nitrogen provides a support for preservation and conservation of this species, thereby facilitating distribution and easy exchange of elite plant germplasm.

1. Introduction

Plants are the principal sources of medicines for human beings since ancient times. Plants have created a dynamic impact on culture, thought as well as on economic activity since ancient period (De, 1980). A vast diversity of plants including medicinal and aromatic plants in addition to weeds has a high medicinal and economic value. Generally, plants that fall under the category of weeds are not given importance due to lack of economic values, and hence its use becomes restricted. One such example of a wild weed that contributes in improving medicinal as well as economic value is *Argemone maxicana* L., commonly known as Mexican prickly poppy which almost grows in arid regions of India particularly Rajasthan (Alam and Khan, 2020). Applications of herbal medicines has gained popularity in the pharmaceutical industries in the recent years because of its ease in availability, safe to use without any side affect and can also be prepared conveniently. In addition, further improvement and extension of herbal drug industries for its quality control along with advances in clinical research are currently molding the general belief in their support (Parveen *et al.*, 2020). Utilization of herbal remedies and healthcare preparations derived from commonly used traditional herbs and medicinal plants has been investigated for extraction of natural products with medicinal properties (Hoareau and DaSilva, 1999). Researchers who had studied the importance of ethnomedicinal uses had considered medicinal plants as an essential source of production of authentic drugs (Chacko *et al.*, 2010). Almost 80 per cent of the populations in developing countries such as India are entirely dependent on herbal medicine for their primary healthcare and nearly 25 per cent of the medicines prescribed by the physicians to the population of developed countries are obtained from wild plant species (Hamilton *et al.*, 2004). Due to the increasing demand in utilization of herbal medicines, natural health products and secondary metabolites of medicinal plants, use of medicinal plants is shed lighted around the

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world (Cole *et al*., 2007). In terms of export of raw materials in pharmaceutical industries, its demand has been tremendously increasing in the country and abroad. In terms of consumption of herbal medicines in an international market particularly Asia and Africa, almost 80 per cent of the population are reluctant in utilizing herbal medicines in a traditional manner in order to get rid of infections and curable diseases, whereas in a country like Japan, 60-70 per cent of the population use 'kampo' drugs which are primarily composed of herbal products. Consequently, in most of the developed countries particularly USA, majority of the population (70-80 %), uses herbal medicines in the form of complementary or alternative medicines (Parveen *et al.,* 2020). Based on the reports of World Health Organization, in countries like Australia, France and Canada; approximately 46%, 49% and 70% of the populations uses herbal products in the form of complementary or alternative medicines. Relatively, 71% of the populations of Chile and 40% of Columbia also utilize herbal medicines. Consumption of raw materials in domestic market has increased more than 30 tons. Because of its increasing commercial demand and price in international markets, overharvesting, premature harvesting and irrational collection practices have been taking place in the natural habitats of the country which is drastically leading to the extinction of the species. *R. serpentina* (Sarpagandha) is one of the primary traded species of the country, contributing an essential component in national and international market. Some of the commercial products of the species are supplied to the companies particularly Planet Ayurveda, Hindustan Mint and Agro Products, Vedic Herbals and Aromatics, Amritanjali Ayurved Pvt. Ltd., Banashpati Ayurvedic Herbal Plants, Dabur Co. Pvt. Ltd., Gorkha Ayurved which are conveniently available in the National and International market. In context to value chain system, it aims in providing specific inputs in the form of raw materials for a particular product to primary production, transformation, marketing and distribution along with final consumption. The chief contributors in the value chain system of *R. serpentina* includes primary collectors, village-level traders, road-head traders, whole-sellers, raw material exporters, herbal enterprises, retailers, consumers as well as input suppliers (ANSAB, 2011). The ever growing requirement of the raw materials from national and international markets has encouraged local communities to accumulate progressively large quantities from their natural habitat in addition to insisting them for instigating growth and promotion of this species in private land, community forests and other normal barren lands. Based on the research findings, cultivation of this particular species has been found to be economically important for the local communities in addition to other parts of the country. Hence, further cultivation of this species is extremely encouraged in order to uplift and strengthen the economic status of the communities of the nation. Based on the importance of medicinal uses particularly Ayurveda, roots of this plant is enriched with astringent and anti-poisonous properties which is useful for curing fever, worm infestations, mental disorders as well as wound healing. Roots are considered to be hypnotic, sedative, anti-hypertensive as well as anthelmintic in nature used for curing ulcers, acts as an antidote to snake bites and scorpion sting madness (Misra, 1993). Apart from it, roots and bark of roots of *R. serpentina* are composed of natural alkaloids particularly reserpine, serpentine, ajmaline, ajmalicine, deserpidine, recinnamine, ajmalicine, serpaajmaline and yohim bine (Rastogi and Mehrotra, 1993). Bark of root is primarily responsible for reducing blood pressure (Manandhar, 2002). In terms of cattle suffering from diarrhea, root extracts are highly valued for intestinal disorders. Leaf juice is frequently in use for cataract elimination of eyes, in addition to its utilization helps in lowering blood pressure. Considering the methods of propagation, it is usually propagated through conventional means particularly through seeds. However, stem cuttings are also practiced to some extent. But, due to the insignificant impact of production leading to lesser percentage of germination in terms of conventional method of propagation in addition to lower percentage of seed viability, lower seed set and scarce or delayed rooting has restricted and limited its conventional method of propagation (Khan *et al.*, 2018). Moreover, seedlings derived from this method are not true to type because of cross pollination. Propagation by means of seeds leads to an introduction of genetic variability which further reduces the occurrence of secondary metabolites including reserpine and other crude alkaloid content, thereby deteriorating its medicinal values. Therefore, in order to prevent from threat of its extinction from further exploitation, an urgent need following suitable strategies of conservation has to be taken into a primary concern.

2. Morphological characteristics

R. serpentina commonly known as devil peppers, snakeroot or sarpagandha is a species of flowering plant and comes under the genus evergreen trees and shrubs in order to honor "Leonhard Rauwolf" in 18th century recognized as a German physician, botanist and traveller whose motive of travel was to be in search of herbal medicines and later on published a set of new botanical descriptions with a herbarium which belongs to the family Apocynaceae. There are around one hundred thirty one species of this genus and amongst them, five of the species are natively grown in India (Kaul, 1956). Five species of sarpagandha (*viz., R. hookeri, R. micrantha, R. serpentina, R. tetraphylla and R. verticillata*) have been recorded in India, of which *R. serpentina* has attained an immense significance as a medicinal plant (Pullaiah, 2006). It is an erect woody shrub attaining a height of about 60-90 cm (Rajbhandari, 2001). Roots of sarpagandha are tuberous in nature consisting of irregularly shaped nodules and pale brown bark attached to it. It attains a diameter of 0.5 to 2.5 cm and grows upto 40 to 60 cm underneath soil. Leaves of *R. serpentina* are arranged in whorls of 3-4 at each node with a length of 17 cm and breadth of 5 cm, resembling simple, elliptical, lanceolate, acuminate, broad, dark green, shiny, thereby tapering into a petiole upto 1 cm (Figure1). Inflorescence is in the form of umbelliferous cyme with bisexual and hermaphrodite flowers depicting white or pinkish colour. It also consists of deep red peduncle of about 1.5 cm long which is actinomorphic in nature with a short pedicel, bracteate having bright scarlet with segments of 2 mm long appearing in clusters. Corolla appears in pinkish colour with salver shaped having a slender tube dilated in the centre with 1-1.5 cm long, literally consisting of 5 lobes particularly indicating the shape of elliptical and oblong with a length of 3-4 mm. Flowers of *R. serpentina* consists of 5 stamens which appears in the form of disc cup, membranous, obscurely lobed and typically inserted at the swollen portion of the corolla tube having irregular corymbose cyme (Deshmukh *et al*., 2012). It is also attached with 2 carpels primarily composed of 2 ovules in each carpel with filiform style and broad stigma, calyptriform at the base and 2 fid at the tip, fruits appears in single drupe or didymous forming an egg shape with pointed ends comprising a diameter of 5 cm which ultimately turns dark purple or blackish when it is completely ripened (Hendrian, 1997). In India, flowers start blooming from the month of beginning of March till the end of May (Uesato *et al.*, 1986). Temperature dependent floral phenology has been reported earlier also by several researchers who conducted diverse studies relevant to floral biology (Sihag and Priti, 1997).

Figure 1: Leaf Morphology of *R. serpentina* (L.) Benth. *ex* Kurz.

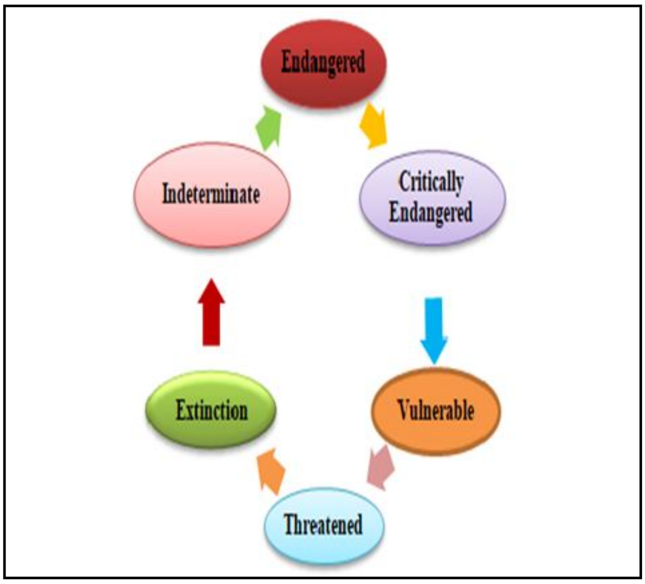

Figure 2: Pictorial representation of IUCN red list threat categories.

3. Geographical distribution

Species of *R. serpentina* was originated in India, Bangladesh, Sri Lanka, Pakistan, Malaysia and Indonesia. In India, this species occurs particularly in foot hills of sub-Himalayan regions upto an elevation of 1300-1400 m, lower ranges of Eastern and Western Ghats, Jammu and Kashmir, North Eastern states of India such as Sikkim, Arunachal Pradesh and Assam (Figure 5), other parts of India such as Chhotanagpur, Uttar Pradesh, Bihar, Orissa, Madhya Pradesh, West Bengal, Maharashtra and Andaman and Nicober Islands (Singh *et al.*, 2009). It is an indigenous medicinal plant native to almost entire parts of India excluding the arid regions.

4. Habitat

Species of *R. serpentina* thrives well in a wide range of diverse climatic conditions but typically prefers hot and humid climatic conditions with a temperature ranging from 10 to 38°C. It prefers to grow well in those regions attributing to high rainfall probably at a range of 1500-4000 mm along with proper drainage system (Sumy *et al.*, 2000). Clay or clayey loam soils rich in organic matter content with a pH ranging from 4 to 8.5 possibly in the range of slightly acidic to neutral soil is found to be most suitable for commercial cultivation of the plant. Presence of high percentage of humus in soils along with organic debris, enabling it to hold humidity levels is highly preferable (Farooqi and Sreeramu, 2001). Usually, the plant thrives well under the shade of forest trees or at the very edge of the forests where three of the four sides are protected against too intense illumination. Favourable environment (habitat) which has been found suitable and is also required by the plant to grow, involves the shade of some woody tree species particularly Sal (*Shorea robusta*), Haritaki (*Terminalia chebula*), Vibhitaki (*Terminalia bellerica*), Asna (*Terminalia alata*), Sisso (*Dalbergia sisso*), Khayar (*Acacia catechu*), and Karma (*Adina cordifolia*) (Anupama, 2016). During extremely winter seasons, the plant starts to shed its leaves since it cannot tolerate lower temperature. Growth of plants are retarded and become prone to root and leaf diseases, if grown on soils containing higher percentage of sand.

5. Global status of species

Genetic resources are presently facing remarkable intimidation due to augmented anthropogenic activities such as deforestation, habitat alteration and unsustainable harvesting for trade. A forest gene bank concept in which a large collection of gene resources is intentionally fabricated and adapted to preserve genetic resources of the *R. serpentina* plant. According to the reports of International Union for Conservation of Nature and Natural Resources (IUCN), species of *R. serpentina* has not yet included in Red List (IUCN, 2006). Based on the information collected in terms of availability of plant species, geographical range, populations and fragmentation of populations of the threatened plants, the International Union for Conservation of Nature and Natural Resources (IUCN, 1995) has divided under the following categories. Pictorial representation of IUCN Red List threat categories have been depicted in Figure 2. But, the same species was listed in the category of "endangered" in southern parts of India covering states like Karnataka, Kerala and Tamil Nadu as well as in central India. As per the information gathered from workshops organised by The Conservation Assessment and Management Plan (CAMP), it has been reported by several researchers stating that the populations of this particular species have gradually decreased more than 50% from census 1985-1995, resulting in loss of habitat due to consumption and utilization by humans in terms of medicinal purpose (Molur and Walker, 1998). Reason behind rapid declination of this species in India having low alkaloid content was due to occurrence of genetic erosion (Ansari, 1993). In the year 1998, another workshop was conducted by CAMP in Bhopal where this same species was categorised as "Critically Endangered" in Maharashtra and the declination rate was observed to be more than 80% from 1988-1998 (Patnaik, 1999). In July 2003, a CAMP workshop was held and the species was assessed as "Critically Endangered" 2001 in states of Chattisgarh, Andhra Pradesh, Maharashtra, whereas on the other hand, in states of Jammu & Kashmir, Uttaranchal, Assam and Meghalaya, it was listed as "Vulnerable" 2001. Most of the populations of this species were threatened due to the collection for medicinal use and trade (Ved *et al.,* 2003). Reports of several researchers after conduction of research stated that the species was categorised as "Vulnerable" in southern parts of India such as Kerala, Orissa and Tamil Nadu (Balakrishna, 1993b). Another workshop on Endangered, Medicinal Plant Species, was held in Himachal Pradesh and listed this species as "Endangered" (Anon, 2002a). It was also been assessed as "Threatened" in India (Ayensu, 1996). In South Eastern Asia particularly Barind tract of Bangladesh, the species had been considered to be "Endangered" (Siddique *et al.,* 2004). Based on the reports of UNEP-WCMC Threatened Plants Database, it has been considered to be "Vulnerable" in Myanmar. But, there is an exception stating that populations are still abundant in a suitable habitat surrounded with moist forest areas and would further decline in those degraded areas (Zaw *et al.,* 2005). Factors which greatly affect the species to be threatened would be change of land use and habitat degradation (Din, 2005). In the year 2001, a CAMP workshop was organized in Nepal confirming that this particular species was assessed as "Critically Endangered" 2001 (Bhattarai *et al.,* 2002) while it was already considered to be in the category of "Extinction" in almost majority of the parts of Nepal (Bhattarai, 1997). Some of the manmade disasters exclusively overharvesting, clearing lands for livestock grazing, cultivation practices such as shifting cultivation and land encroachment for cultivation had led to threaten the species (Amatya *et al.,* 2005). Based on the collection of reports from CITES Scientific Authority, Nepal's CITES Management Authority concluded that it has been categorized as "Threatened in the Wild" in Nepal (Sharma *et al.,* 2006). However, Forest Act 1993 and CITES 1975 have taken steps in order to provide a legal protection from over harvesting of this species. In Sri Lanka, its status has been reported as "indeterminate" (Wijesinghe *et al.,* 1990).

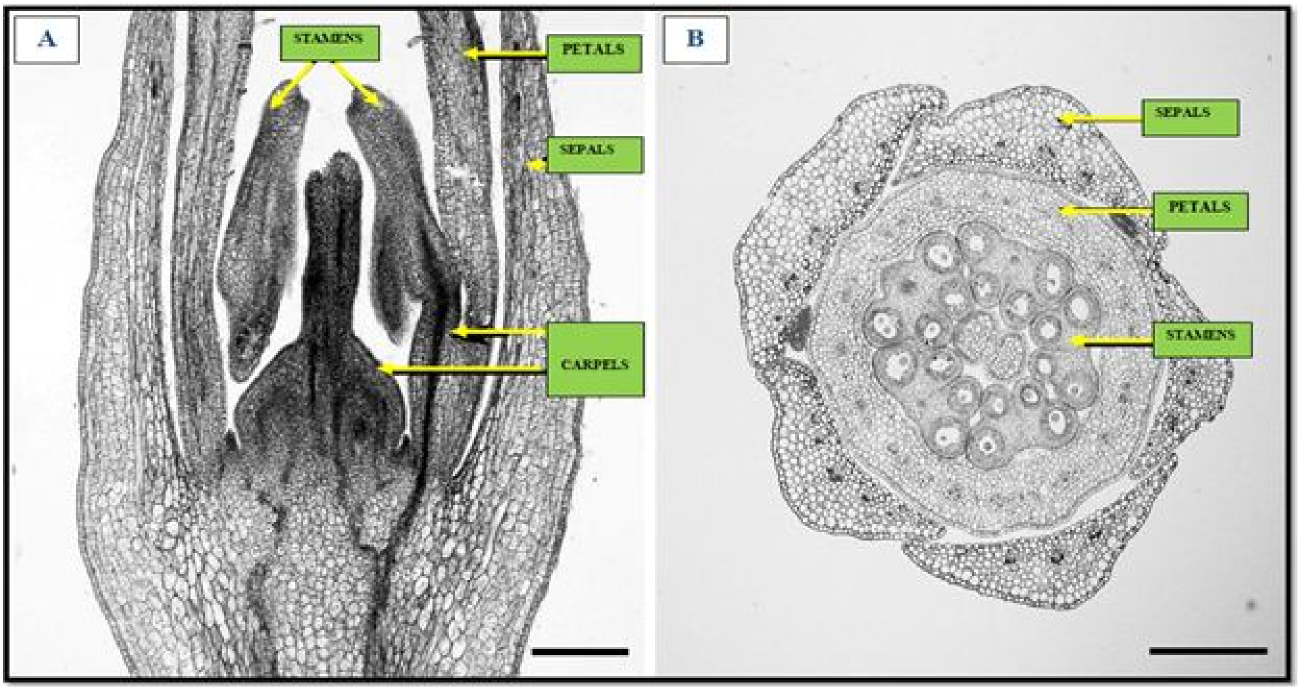

Figure 3: Longitudinal section of young flower of *R. serpentina* indicating sepals, petals, stamens and carpel (B) Transverse section of young flower of *R. serpentina* with diverse parts arranged in whorl. Scale bars indicate 100 pin in Figure 1A and 50 pin in Figure IB. (Source: Ghimire *et al*., 2011).

Likewise, this species was considered to be under the category "Endangered" in Vietnam (Phan Thuc Vat, 1996). The main purpose of organizing Conservation Assessment and Management Prioritization (CAMP) workshops in collaboration with many government and non-government organizations, research and educational institutions, state forest departments and various medicinal plant stakeholders in different states across the country was to identify and prioritize medicinal plants which are already facing towards the category of endangered, critically endangered, threat of extinction in wild and vulnerable. The IUCN Red List categories are intended to be easily and widely understood system for classifying species at high risk of regional to global extinction and to provide an explicit and objective framework for the classification of species according to their extinction risk (Express News service, 2019). Export of products manufactured from this species without processing has been entirely banned by the Nepal Forest Act 1993, Forest Regulation 1995 and Amendment 2001. Ministry of Forest and Soil Conservation and Department of Plant Resources, Kathmandu, Nepal have included the species in national priority list for cultivation purpose (DPR, 2012). Hence, this medicinal plant has been listed as protected plant in Nepal. Based on the data collected with respect to climate and altitude from field surveys, visit to herbarium centre, which were subjected to DIVA GIS in order to plot a predictive map of the plant habitat, it represented that western Terai region was found to the safest zone for this species (Kunwar, 2019). Currently, products of *R. serpentina* manufactured from roots are adulterated with wild plants since suppliers from wild sources are limited; hence due to an increase in rate of exploitation, it has hindered in continuation of constant and steady supply. Consequently protection of forest based genetic resources has turned out to be a global concern which urges expansion of effective conservation strategies at local, national and international level. In order to conserve the threatened plant species having medicinal purpose, several strategies for policy makers and programmes for conservation and utilization of these resources in future generations, proper harvesting by reduction of further exploitation, cost effective scientific cultivation methods, processing, marketing of raw materials in addition to several policies have been developed by the institution particularly National Medicinal Plants Board. In terms of conservation of these highly valuable medicinal plant species for long term storage at NBPGR (National Gene Bank), a Memorandum of Understanding (MoU) has been signed on 6th of July 2020 between ICAR, NBPGR and NMPB (ICAR, NBPGR, 2020). In future, a collaborative project needs to be carried out for *ex situ* conservation of *R. serpentina* species and a database should be prepared at national and international level with major outcomes of the project. In addition, gaps should be critically examined in terms of research works that are being carried out on this particular species. Hence, for long term safe storage and conservation of this species, cryopreservation could also be an alternative option. Utilisation of medicinal plants particularly *R. serpentina* in India exhibits several healthcare benefits, and hence encourages in manufacturing of health-care products. In the recent years, the demand for utilization of medicinal plants and its raw materials particularly *R. serpentina* is increasing rapidly in national and international market because the cultivators are generating employment in addition to income from these plants throughout the year. Therefore, there is an urgent need to conserve this highly demanded threatened medicinal plant by emphasising effective policies on several aspects particularly in terms of cultivation, education, capacity building, research, regulations and trade in addition to conservation. Apart from its policy making aspects, there is also an urgent requirement of creating awareness by popularization of this highly valuable medicinal plant through the use of press reports, publications of the findings by the researchers, advertisement, education as well as scientific reports. These challenges cannot be expected to be accomplished by a national market particularly India, but is also expected to be met by the International market worldwide in collaboration with the stakeholders, harvesters, manufacturers, government agencies, non-governmental organizations and researchers, *etc*. Currently, several organizations that are involved in collection and conservation of these species for future generations and are under trial with several projects globally includes TRAFFIC, BFN, IUCN/SSC Medicinal Plant Specialist Group (MPSG) and WWF Germany (International Standard for Sustainable Collection of Wild Medicinal and Aromatic Plants, ISSC-MAP) (Divya *et al.,* 2018).

6. Floral biology and pollination efficiency

Floral biology is a branch of ecological research that studies the evolutionary factors so as to mould the structures, behavior and physiological aspects involved in the flowering of plants. A slightly narrower area of research within floral biology is sometimes termed as pollination biology (Mary, 2013). The primary goal to study floral biology is to emphasize on functional role of flowers in promoting pollination and mating. Populations of an absolute organism in the ecosystem are tremendously important to normalize and stabilize the ecosystem (Odum, 1971) which is extremely apparent in case of pollinator plant systems too, where large numbers of pollinator species are at hand. Relatively, diverse populations of insect pollinators are enormously essential in controlling and stabilizing the pollination procedure of entomophilous plants and hence, in terms of mating system, research has been predominated by population genetic and theoretical perspective with little consideration given to the proximate ecological factors responsible for causing a particular pattern of mating to occur (Lloyd, 2012). Understanding of reproductive biology of a plant is a must for attempting any breeding programme (Singh *et al.,* 2010). Because of high demand of medicinal properties of *R. serpentina*, it has proven essential to study about floral biology which would further become beneficial for hybridization programme. Furthermore, information regarding floral biology is a prerequisite in assessing overall reproductive potential of the species. Flowers of *R. serpentina* are literally composed of narrow and long tubular corolla (Figure 3). Based on the study of previous reports confirmed by several researchers, it has been stated that such flowers are highly beneficial for initiating psychophily pollination syndrome negating all other syndromes (Sihag and Kaur, 1997). Consequently, stigma receptivity is the ability of stigma to sustain viable and compatible pollen to germinate. Time and period of stigma receptivity should be accompanied for successful breeding of crops (Stone *et al.,* 1995). Usually at the time of anthesis, the stigma is receptive and may lose for one, two or several days (Kalinganire *et al.,* 2000). Based on the experiments conducted so far in terms of pollination and receptivity of stigma, it had been concluded that stigma was receptive only on the first day in age of two days flower since anthers were dehisced on the second day when stigma had become non-receptive (Sihag and Wadhwa, 2011). Results were also in conformity with the reports of several researchers stating that flowers of *R. serpentina* are required to be cross pollinated as in umbelliferous plants (Sihag, 1985a), onion (Sihag, 1985b) and various cultivars of litchi (*Litchi chinensis* Sonn) (Ray and Sharma, 1995) collected from newly born inflorescence of *R. serpentina* were utilized to carry out the experiments in order to conduct work on phonological studies and crossing program in the year 2007-2009 in Institute of Forests Genetics and Tree Breeding, Coimbatore and it was critically observed that new buds had started blooming in 4-6 weeks with an opening rate of 4-25 flower buds per day which further continued upto 60 days. Fruits were found hanging till next flowering season until dispersed by gravity (Usmani *et al.,* 2015). These results were also in agreement with the reports of Faegri and Pijl (1979). Pollination is one of the most important mechanisms for preservation and conservation of biodiversity in broad-spectrum life of earth. Pollinators are endowed with an ecosystem service that enables plants to breed fruits and seeds. More than 80 per cent of total pollination actions are performed by insects and bees (Klein *et al.,* 2007). The arrangement of flowers, their extent of self-fertility and their arrangement on the plant determines the scope to which a plant is reliant on insects for pollen transfer (Devi *et al.,* 2015). Flowers of angiosperms catch the attention of an extensive diversity of visitors, the definitive desirability is the floral reward comprised of nectar and pollen. The sweet floral satisfying the nectar is the most important attractant and its arrangement pattern in flower determines how easily it is accessible to a seeker (Abrol and Sihag, 1997). Pollinating agents particularly those insects which help in the process of pollination during flowering period of a plant in the form of visitors would consequently aid in identification and differentiation of pollinators and non-pollinators. It is thus of immense significance to differentiate between pollinating and non-pollinating visitors. As per the reports concerned so far, there are certain aspects which help in further control and management of pollinating potential of flower visitors particularly insects (Narwania *et al.*, 2003). Indeed the most excellent pollinator is one that realizes greatest reproductive potential in the plant. Visitors' performance subsequently has immense behaviour on reproductive achievement of the plant it visits. Approximately 30 per cent of human food is obtained from bee pollinated crops (Toole, 1993). Pollinating insects are extensively originated in insect orders such as Coleoptera, Lepidoptera, Thysanoptera, Diptera and Hymenoptera (Singh and Garg, 2003). Based on the findings of Anoosha *et al.* (2018), flowers of *R. serpentina* attracted 17 species of insects and amongst them, Lepidopterans were found to be the major floral visitors comprising from three families, *viz*., Papilionidae (*Papilio demoleus* and *Papilio polytes*), Pieridae (*Eurema hecabe*, *Pieris* sp., *Anaphaeis* sp., *Pieris canidia*, *Belenois aurota*, *Pieris brassicae* and *Colotis etrida* and Hesperiidae (*Pelopidas* sp.). They were belonging in order of uniformity by Dipterans from two families', *viz*., Sarcophagidae (*Sarcophaga* sp.) and Syrphidae (*Eristalinus obscuritarsis*) and two species from two families of Hymenoptera, *viz*., Vespidae (*Polistes olivaceus*) and Apidae (*Amegilla zonata*) and Coleopteran from family Coccinellidae (*Coccinella septempunctata*). Out of 17 insects, all were top foragers except *A. zontata* which was side forager. These conclusions are in conformity with those of Wadhwa and Sihag (2012) who recorded 19 insect species visiting the flowers of *R. serpentina*. *P. demoleus* was usually a rich pollinator and was also found in large quantity in terms of insect visitors of species *R. serpentina* which were observed to be low at the time of opening and closing of the flower and high during peak flowering period (Wadhwa and Sihag, 2012). These informations were in line with findings of Anoosha *et al*. (2018) confining that the highest population of all major insect pollinators were recorded during 2nd week of flowering which was drastically different with the flowering period of 1st week and 3rd week. Pyke *et al.*(1977) assured that during the period of peak flowering, additional number of pollen and nectar were available which concurrently attracted more pollinators and further equivalent conclusions were recorded in the study conducted by Anoosha *et al.* (2018). Hence, based on the results of the experiments carried out so far, it can be assured that Psychophilous mode of pollination is prevalent in the species *R. serpentina* due to its tubular flower structure and therefore, conservation of these pollinators is a must for preventing the threatened species from further extinction. Seed and fruit production in plants depends upon several factors and inputs (Kumar *et al.*, 2011). For managed pollination of a plant, there is a fundamental need to be acquainted with its pollinators. In case of fruit set, under open pollinated conditions, lower fruit set was observed in *R. serpentina* which was also occurred in many tropical trees (Bawa, 1974). It has been proven that limitation in pollination is mainly due to lower fruit set (Calvo, 1990). Under self pollinated condition, flowers yielded lower fruit set (3.9%) while open pollinated flowers showed highest fruit set (21%) whereas cross pollinated flowers showed 12% fruit sets which clearly indicated that species of *R. serpentina* is completely a cross pollinated flower (Usmani *et al.*, 2015). Similar results were also recorded in *R. grandiflora* stating that species is literally a cross pollinated with sporophytic self-incompatibility system (Lopes and Machado, 1999). Inhibition of self pollen is frequent in the species *R. serpentina* because of its sporophytic mode of self incompatibility and generally occurs at pollen stigma interface (Newbigim *et al.,* 1993). Another report on floral phenology confined that occurrence of flowering in *R. serpentina* was observed through the entire year but blooming and anthesis greatly varied with summer and winter season because of some abiotic factors such as photoperiod, light intensity, temperature, moisture supply with ambient humidity, soil moisture and nutrient supply (Akare and Chaturvedi, 2018). For proper germination of pollen, stigma provides a path but studies on *in vivo* nature of pollen germination have not proved to be successful because of obstacles involving in pistillate tissue (Biswas and Mondal, 2014). Another report on evidence of cross pollination was found on the same species indicating highest rate of viability of pollen grains and stigma receptivity at the time of anthesis ensuring maturation of pollen grains and stigma at the same time which clearly resembled occurrence of self-incompatibility in flower, thereby leading to cross pollination (Akare and Chaturvedi, 2018). The results obtained from species of *R. serpentina* were also found to be similar in case of species *R. micrantha* (Kulloli and Sreekala, 2009).

7. Conservation strategies

A variety of recommendations attributing to conservation and protection of medicinal plants have been developed, such as providing mutually *in situ* and *ex situ* conservation, natural reserves and wild nurseries are distinctive examples to preserve and maintain the medicinal efficacy of plant life in their natural habitats and at the same time, botanical gardens and seed banks are essentially beneficial intended for *ex situ* conservation, preservation in addition to further utilization for future generations (Cole *et al.,* 2003). Geographical distribution and biological descriptions of medicinal plants are ought to be identified to guide conservation activities, such as to decide whether species conservation have to take place in natural habitat or in a nursery. There is an urgent requirement to conserve germplasm of thousands of rare, elite and endangered species of medicinal plants particularly those of recalcitrant types where in seed cannot be preserved. Considerably, *in vitro* culture has played an important role in which micropropagation studies can be effortlessly conducted (Bajaj, 1995). Advancement of genetic engineering has led to the possibility of extensive biosynthesis of natural products along with advancement in tissue culture and fermentation of medicinal plants that have opened new avenues for manufacturing of important and highly proficient advantageous bioactive compounds. Tissue culture (including plant cell and transgenic hairy root culture) is a promising alternative for the production of exceptional and high-value secondary metabolites endowed with medicinal importance (Rao *et al.,* 2002). Micropropagation *via* tissue encapsulation of propagules is being capable of not only facilitating storage and transportation, but also promotes higher regeneration rates (Baker *et al.,* 2007). Once, the amount of normal seeds tends to be deficient for propagation, synthetic seed technology, specifically defined as artificially encapsulated somatic embryos (or other tissues) could be utilised for propagating either under *in vitro* or *ex vitro* conditions which proves to be a possible choice (Lata *et al.,* 2008). In addition, breeding improvements can be practised using molecular marker-based approaches functional at the genetic level, and the time necessary for breeding perhaps can be considerably shortened (Lata *et al.,* 2008).

8. Optimum temperature and moisture content for storage in seed banks

For conservation and preservation of genetic diversity in seed banks, drying of seeds to optimum moisture content as well as for determination of genetic purity and quality evaluation in order to store it in moisture proof containers is under necessity. Storage of seeds of diversity either in seed banks or gene banks seems to be the safest and is proven to be one of the most convenient and inexpensive method for conservation since it requires less space and low maintenance over a considerable period of time (Engelmann and Engels, 2002). Because of an adverse affect occurring in the form of biotic and abiotic factors, there is a high probability of risk in terms of conservation of endangered medicinal plants germplasm under an external environmental condition. However, storage of seeds in seed banks under *ex situ* conservation requires an attention in terms of some important factors like post harvest seed physiology and seed storage biophysics as seeds that comes under the category of orthodox seed storage behaviour which can be dried to 5-7% moisture content having a desiccation tolerance mechanism would likely to be stored in seed banks for long term storage without losing its viability. Relatively, desiccation tolerant seeds can be quantified by seed viability equations, as seed viability depends upon chemical composition of seed, moisture content and storage temperature (Hong *et al.,* 2005). Till date, very limited information is available related to the factors like seed morphology, dormancy and seed storage behavior which would further help in contributing to conservation and storage of seeds on seed banks. Hence, in order to get a thorough understanding on the same, a research was conducted with seeds of *R. serpentina* by collecting its matured seeds from Sushila Tiwari Herbal Garden, Rishikesh, Uttarakhand, India in which germination test and seed viability test using topographical tetrazolium test were carried out with respect to desiccation and chilling tolerance using a factorial combination of four moisture contents (5, 7, 10 and 12% moisture content in seeds on fresh weight basis), three storage temperatures (seeds stored in ambient condition, at 15°C and at –20°C) in addition to storage for a period of 0-12 months particularly at an interval of 3, 6, 9 and 12th months. Based on the results revealed, it was confirmed that there was no loss in germination of seeds with presence of 7% and 10% moisture content under ambient as well as other storage conditions, signifying that seeds of *R. serpentina* comes under the category of desiccation in addition to chilling tolerant and exhibited orthodox seed storage behavior (Choudhary and Chauhan, 2015). Hence, it could be concluded that seeds were perfect for *ex situ* conservation in seed banks or gene banks for a longer period of time.

9. Methods of propagation

Species of *R. serpentina* can be propagated by several methods; for example seeds, root cuttings, root stumps and stem cuttings. However, conventional propagation methods of this plant face some major constraints indicating poor seed viability and germination. Under these conditions, application of *in vitro* conservation methods such as *in vitro* culture and micropropagation along with synthetic seed production in addition to techniques of cryopreservation provides a support for preservation and conservation of this endangered species. Moreover, it facilitates distribution and easy exchange of elite plant germplasm (Naik and Chand, 2006).

9.1 Propagation through Seeds

Seeds of this particular species when sown directly in the field does not show any successful rate of germination and, therefore it is recommended to propagate through seedlings raised in the nursery bed and later on transplanted in the main field. Due to an adverse influence as well as presence of stony endocarp, there has been a decline in the rate of germination (25-30%) and would often decrease upto10%. Another possible reason for low rate of germination might be due to devoid of embryos in fruits leading to parthenocarpy or of deferred somatoplastic sterility. Reports on recent studies have indicated that highest germination percentage (58-74%) was obtained from fresh seeds of ripened fruits collected immediately before sowing (Amatya *et al.,* 2005). A delay in collection and sowing of seeds within 24-36 hours as well as sun dried and stored seeds would definitely reduce the rate of germination. Seeds stored for more than 7-8 months did not germinate at all due to loss in viability. In order to retain viability of seeds for at least six months, ripened seeds were collected in the month of June and continued till the end of October to November and stored in air tight containers. Propagation of *R. serpentina* through seeds was first reported by Nayar (1956). Seed propagation is considered to be the best method for raising commercial crop, though seed production is highly variable and low (Bhadwar *et al.,* 1956).

9.2 Vegetative propagation

Vegetative propagation by root and shoot cuttings are being mainly prioritized for raising plantations since collection of seeds from wild sources is laborious and costly. First report on vegetative propagation of plant was studied by Chandra (1954) whereas Hedayatullah (1959) reported culture and propagation of this plant. For propagation by root cuttings, large tap roots with a few filiform lateral secondary rootlets are used. Consequences on propagation by root cuttings practiced in some places such as Dehradun had shown that root cuttings of about 0.25 cm diameter planted under irrigated conditions during the month of March-June had given a success rate of 52-79%. Development of healthy roots along with shoots started occurring right after 10-15 days of propagation. Higher percentage of flourishing plants obtained by root cuttings makes it profitable to propagation by seeds. On the other hand, alkaloids content of plants derived from roots were found to be less as compared to the plants raised from seeds. Vegetative propagation through root cuttings was mainly encouraged because of lower rate of germination and poor seed viability due to the presence of cinnamic acid and compounds available in the seed (Mitra, 1976). Method of propagating through root stems was obtained by planting 5 cm of root with a portion of stem above the collar. A success rate of about 100% was obtained using such method of propagation. Apart from its advantages, it has its limitations such as only an individual plant can be raised from a single stump. Another method of propagation such as stem cuttings including hardwood and softwood cuttings of 15-23 cm long consisting of 2-3 internodes were found to be most suitable. Based on the investigation of reports, it had been found that hardwood cuttings treated with 30 ppm of indole acetic acid solution had given maximum percentage of rooting (60-100%) within a period of 15 days. However, stem cuttings have been found to be less satisfactory than root cuttings since most of the plants obtained through stem cuttings do not start rooting easily.

9.3 Synthetic seed production

Plant cell and tissue culture is a challenging technique for extraction of phytochemicals under controlled conditions of environment irrespective of external conditions. Additionally, proper selection of source of explants is essential for proficient production of phytochemicals as biosynthesis and accumulation of phytochemicals under the influence of environment is tissue or organ specific (Sharma *et al.*, 2021). Production and development of synthetic seeds leads to the advancement in plant biotechnology. Purpose of this technology has recently been equipped for plant life ranging from herb to tree to resolve inconvenience arising out of storage, viability and germination of plants' natural germplasms propagated by seeds or vegetative parts (*viz.*, corm, bulb, rhizome, *etc.*) implementing as seeds. The alginate coat-protected somatic embryos (Malabadi and Staden, 2005), shoot tips or nodal segments (Naik and Chand, 2006), axillary buds, micro-plants or cuttings (Tsvetkov and Hausman, 2005), and roots having regeneration potential (Brischia *et al.*, 2002) are all integrated under the category of synthetic or artificial seeds. As for the reason that somatic embryogenesis does not occur in all of the cultured plant species, encapsulation of *in vitro* derived tissues and organs, in particular as well as utilization of shoot tips and nodal segments as beading material has gained additional popularity and ensured further wide spread application of this technology in storage, exchange and mass propagation of plant species. Alginate encapsulation method is intended to combine the advantages of clonal propagation with those of seed propagation and storage (Ara *et al.*, 2003). In case of explants such as nodal segments encapsulated in 3% (w/v) sodium alginate and 100 mM calcium chloride accurately stored at 4°C and placed on woody plant medium supplemented with 5.0 µM BA and 1.0 ìM NAA showed emergence of shoots after 2 weeks of incubation. After four weeks of storage at 4°C, percentage conversion of encapsulated nodal segments into a complete plantlet was 80%, whereas, non-encapsulated nodal segments produced about 21% plantlets (Faisal, 2012). Further, these encapsulated and non-encapsulated nodal segments were transferred into a fresh medium containing BA (5 µM) and NAA (1.0 µM) for acclimatization. Meanwhile, comparison of different basal medium fortified with varying concentrations of plant growth regulators were also examined to detect best medium suitable for maximum shoot regeneration derived from encapsulated nodal segments following cold storage at 4°C for four weeks. Murashige and Skoog in 1962 (MS), WPM, Gamborg *et al.* in 1968 (B5) and Schenk and Hildebrandt in 1972 (SH) medium were used with 5.0 µM BA and 1.0 µM NAA. Results obtained from the experiment conducted with cold-stored encapsulated nodal segments were in conformity with the study of Faisal *et al.* (2006) pertaining to the rates of encapsulated segments with axillary buds of *R. tetraphylla* stored at 4°C. For encapsulation process in *R. serpentina*, explants such as axenic shoot tips and nodal segments of size 3-5 mm were excised from *in vitro* raised seedlings and those encapsulated propagules were further cultured in MS media containing 3% sucrose without any addition of plant growth regulators and incubated under temperatures @ 4°C, 12°C and 20°C. Shoots that had derived from pierced matrix wall of synthetic seeds were treated with rooting hormone consisted of MS media supplemented with IAA, IBA and NAA @0.5–2.0 mg/l. Seedlings with well grown roots and shoots were further transferred to the hardening chamber in order to develop into a complete plant (Ray *et al.*, 2008). Based on the storage of synthetic seeds in terms of temperature and duration in days, it has been confirmed that synthetic seeds stored at 25°C for 30 days had showed higher rate of germination (82%), whereas on the other hand, graph of germination percentage declined when storage was done at 8°C, but improved reserpine content of synthetic seeds (Gantait *et al.*, 2017b) (Figure 4). Regarding dropping of encapsulated beads on calcium chloride solution at varying concentrations, use of 100 mM $CaCl_2$ was found to be suitable (Faisal *et al.*, 2013) while in another report, it was concluded that 75 mM of $CaCl_2$ was observed to be more suitable for synthetic seed production in *R. serpentina* (Gantait *et al.*, 2017b). Further, it was cultured into regeneration media consisting of BA and NAA for shoots regeneration. Those regenerated microshoots were again cultured into rooting hormone (IAA + IBA) for occurrence of roots for conversion into a complete plantlet. Later on it was acclimatized to grow under field conditions (Faisal *et al.*, 2013).

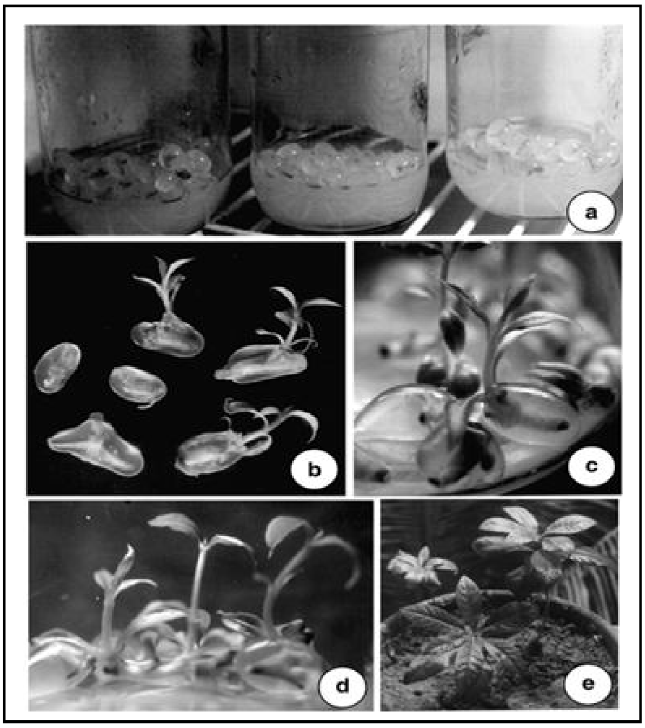

Figure 4: **(A) Storage of synthetic seeds of *R. serpentina* at 4°C, (B) Various stages of germination of synthetic seeds, (C) Initial stage of *in vitro* shoot formation, (D) Plantlets obtained from synthetic seeds and (E) Acclimatized plantlets obtained from synthetic seeds (Source: Ray and Bhattacharya, 2008).**

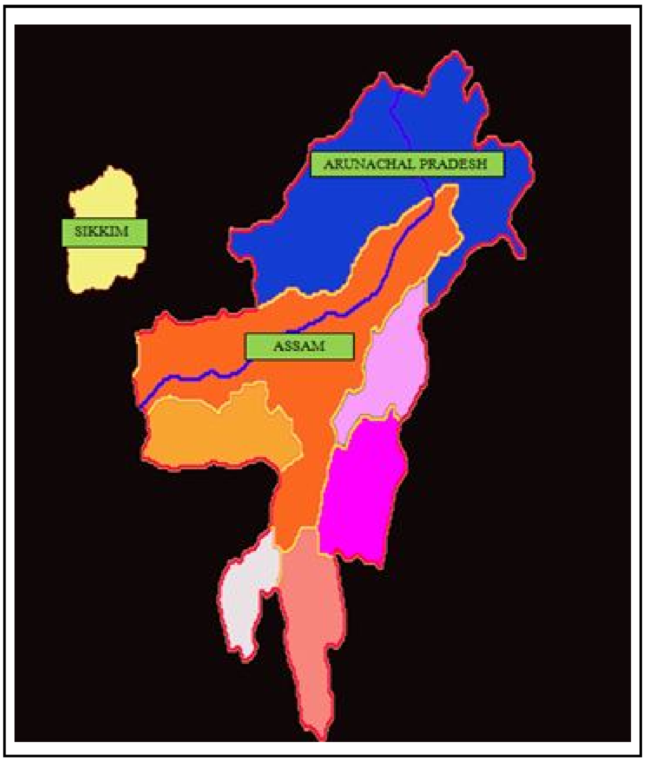

Figure 5: Distribution of *R. serpentina* under North Eastern India.

9.4 Application of cryopreservation

The main objective of cryopreservation is to store planting materials particularly seed, pollens, somatic and zygotic embryos at ultra low temperature in liquid nitrogen (–196°C) or in its vapour phase (–150°C). The technique of cryopreservation had been developed since last two decades ago with an objective of long term conservation of endangered species of flora and fauna. Cryopreservation method has been applied successfully using several plant genotypes. Some of the techniques that have been applied include encapsulation, vitrification, encapsulation-vitrification, droplet freezing technique, pre-growth, desiccation as well as combination effect of pre-growth desication (Engelmann, 2004). Application of these methods helps in reduction of somaclonal variation since the process of sub-culturing the explants becomes limited. In addition, it is a low cost effective method which helps in storage of germplasm including plant cells and tissues for a longer period of time. Cryopreservation techniques are specifically applied in vegetatively propagated crops suitable for long term storage in addition to other genetic resources (Kaczmarczyk *et al.,* 2008). Development of simple cryo-storage protocols for orthodox seeds has allowed cryopreservation of a large number of species at low cost, significantly reducing seed deterioration in storage (Stanwood, 1987). Based on the results obtained by several researchers so far, limited work has been carried out in terms of cryopreservation of seeds of wild and endangered species of plants in addition to medicinal plants (Rajasekharan, 2006). Tropical Botanic Garden and Research Institute (TBGRI), Trivandrum has significantly developed cryopreservation protocol on rare and endangered medicinal plants of India (Radha *et al.,* 2006). A cryo-bank was also established which now holds more than 25 accessions of medicinal and aromatic plants (Radha *et al.,* 2010). For successful PVS2 cryopreservation of *R. serpentina,* nodal segments and shoot tips of three different sizes were excised for conduction of the experiment. Based on the results, it was confirmed that 0.31-0.39 cm nodal segments, pre-cultured for 7 days in 0.5 M sucrose at 4°C under diffuse light, loaded for 30 min and 45 min in PVS2 had given maximum viability (66%) after cryopreservation (Ray and Bhattacharya, 2008). Very few works with respect to this species was carried out earlier; most of these had been on seeds and application of procedures to medicinal plants is still very limited. Another report on cryopreservation was obtained involving encapsulation of 200 numbers of hairy root tips of around 2-3mm in 3% w/v sodium alginate. Excision of root tips was derived from *in vitro* grown 4-6 weeks old hairy root cultures maintained in B5 medium. Root tips encapsulated sodium alginate beads of size 68 mm were further experimented for sucrose pre-culturing (0.5M sucrose for 24 h) and dehydration for 6 h. Meanwhile, encapsulated beads were sealed in cryovials consisting of 12 beads each and directly incorporated into liquid nitrogen for 7 days. Those beads were further removed and kept under normal conditions at around 35°C for 5 min which later on cultured in semi solid B5 medium. After 45 days of culture, 40% of the cryo-preserved root tips exhibited re-growth which was entirely due to the impact of pre-culturing that functioned as a dehydration, thereby leading to an improvement in re-growth potential of encapsulated root tips (Mehrotra *et al.,* 2015).

10. Conclusion

Although, species of *R. serpentina* can be propagated by several methods such as seeds, root cuttings, root stumps and stem cuttings; however, conventional propagation methods of this plant face some major constraints indicating poor seed viability and germination. Based on the collection of reports confirmed by several researchers on various aspects of this endangered medicinal plant *R. serpentina,* it can be clearly concluded that this particular species is in a threat of extinction due to overexploitation by humans in terms of consumption for their medicinal values and hence, there is an urgent need to protect and conserve this species for mass multiplication leading to increase in plant population. Hence, in order to conserve this species, study of floral biology and its pollination efficiency has become a necessity since it would help in further understanding of its fertilization potential as well. Moreover, conservation strategies such as *ex vitro* in the form of seed banks, natural reserves, wild centuries, botanical gardens, *etc.,* would benefit this species from threat of extinction. Considerably, *in vitro* conservation methods in terms of advanced biotechnological tools such as tissue culture which has been proven to be a highly promising alternative for production and mass multiplication of endangered medicinal plants endowed with natural secondary metabolites, slow growth root culture, *in vitro* micropropagation (capable of not only facilitating storage and transportation, but also promotes higher regeneration rates), production and development of synthetic seeds along with preservation of seeds, shoot tips, embryos, nodal segments in liquid nitrogen at –196°C or in a vapour phase at 150°C without losing its viability preferably known as cryopreservation would prove to be beneficial for long term storage as well as conservation and utilization of germplasm which are in a threat of extinction. Moreover, in case of synthetic seed production, encapsulation of *in vitro* derived tissues and organs, in particular as well as utilization of shoot tips and nodal segments as beading material has gained additional popularity and ensured further widespread application of this technology in terms of storage, exchange and mass propagation of this endangered medicinal plant.

11. Future prospects

In vitro conservation by means of meristem and shoot tip cultures consisting of least media at lower temperatures along with lower intensity of light is considered to be the most promising alternative which apparently provides satisfactory methods for germplasm conservation. Consequently, cryopreservation of seeds, pollen and *in vitro* cultures would put forward an exceptional method intended for preservation and utilization of germplasm. These can be proven to be extremely beneficial for vegetatively propagated crops, plant species endowed with recalcitrant seeds, wild and endangered species and also wild relatives of crop plants. Relatively, method of cryopreservation can be considered to be a potent method since more than 50 species have been effectively freeze preserved. Keeping in view regarding its fruitful impact of cryopreservation, it could be efficiently utilized in storage of plant tissue cultures bestowed with essential biochemical and morphological characters in addition to long-term storage of tissue culture collections in any repository. Moreover, occurrence of somaclonal variations allied with definite culture system in any *in vitro* conservation programme is required to be taken care of. Apparently, there is an urgent need to create a well-built relationship among institutions and centers of excellence specifically concerned into research on diverse aspects of genetic conservation of endangered medicinal plants. In recent years, gene cloning technology has led to fundamental advances in multiple areas of ecology. Application of cloned fragments of chromosomal DNA as genetic markers, typically termed 'RFLPs' (Restriction Fragments Length Polymorphism) has achieved considerable importance. This innovative technology promises to transform several areas of plant genetics, crop breeding and genetic conservation into a next advanced level of biotechnology. Ultimately, a range of approaches discussed in this manuscript would definitely make available a functional principle of creating attentiveness among scientific population as well as would contribute extensively towards establishing conservation programs predominantly in gene rich countries of the entire world in the right perspective.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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