1. Introduction

1.1 General Introduction

Conservation and maintenance of plant diversity is of supreme importance and global interest. Rapid increase in human population and a number of men-made activities such as deforestation, amendments in agricultural practices, industrialization, urbanization and changes in land-use are severely disturbing the plant diversity, which results in the increase in the number of threatened species. In addition, introduction of alien species, weed infestation, etc., also imposes dramatic impacts over plant diversity (Rao 2004). Loss of plant diversity jeopardizes, resilience in ecosystems, including the ability to respond to the stresses brought about by climate change, thereby diminishing possible re-establishment of species after the disasters (Abbas and Qaiser 2011). Over exploitation of plant resources for day-to-day requirements, does not only contributes loss of diversity but also limits the availability of basic raw materials and genetic variability, which is very important in breeding programs for developing more productive crops and more resistant plants to biological and environmental stresses (Ohmiya et al. 2003).

Plant diversity can effectively be conserved following either in situ or ex situ practices or both. The preservation of a particular plant species in their natural habitat or maintenance of cultivated species in the gardens or farms represents the in situ strategies (UNCED 1992). However, due to habitat destruction and the transformations of natural environments of plant species, there is a heavy loss or decline in their populations, which can lead to a loss of diversity. Therefore, in situ methods alone are not enough for conserving threatened taxon. Additional approaches like botanical gardens, storage in seed banks and in vitro preservation procedures complement the conservation programs. These techniques are classified as ex situ strategies, which stand for maintaining the biological material in another place instead of their natural habitats (Engelmann 2012). Ex situ technique is a feasible way for conserving plant diversity from extinction, and in some cases, it is the only possible approach for saving certain species (Ramsay et al. 2000). Both in situ and ex situ strategies are complementary to each other as they offer various alternatives for conservation, but the selection of the appropriate method should be based on a number of criterion such as biological nature of the particular species, feasibility of applying the chosen strategy, etc. (Engelmann 2012). Advances in plant biotechnology, particularly those associated with in vitro culture, has also provided
tremendous tools to improve conservation and management of plant diversity (Withers 1995). Now-a-days, biotechnological applications have been used to conserve the threatened species along with the taxon of commercial importance such as ornamental, crop, medicinal and forest species. These techniques allow conservation of pathogen-free material and elite clones. Moreover, it is also able to maintain the genetic diversity for short, medium and long-term durations. *In vitro* conservation strategy is particularly important for vegetatively propagated plant species (Engelmann 2011). Furthermore, these techniques offer a safe mode for international exchange of plant materials as well as allow the supply of valuable materials for the recovery of wild populations (Tandon and Kumaria 2005).

1.2 Conservation Practices

The conventional method of germplasm conservation includes their maintenance as whole plant in the field (Pathirana et al. 2016). Field maintenance of plant materials not only carries the risks of infections of viral, fungal, bacterial diseases and insect-pests, but also includes losses due to the environmental disasters such as flood, earthquake, drought, fire, volcanic eruptions, etc., which has led to the erosion of valuable germplasm resources (Barba et al. 2008; Carimi et al. 2011). However, duplication of materials in different fields is an option, but is a quite expensive approach. Therefore, risks involved in field maintenance have led us to search for secure, cost-effective and efficient protocols for effective conservation of plant diversity.

The major obstacles in *in situ* conservation practices are the requirement of larger space, high cost of operation, complicated management, and risk of damage by both biotic and abiotic factors of the environment. Thus, an *ex situ* strategy shows efficacy towards the preservation of plant genetic resources over *in situ* methods. The plant genetic resources are conserved in the forms of seeds, bulbs or tissue culture derived propagules in various gene banks (Paunescu 2009). Among these, the most suitable method suggested for long-term *ex situ* conservation of any species is storage of their seeds. However, in case of vegetatively propagated and a species with low germination rate/ viability, storage of seeds is not a suitable option. In such cases, *in vitro* preservation is a successful method for both mid-term and long-term storage (Engelmann and Engels 2002; Theilade and Petri 2003; Uyoh et al. 2003; Rao 2004).
In addition, another importance of *ex situ* conservation is that it is an internationally accepted strategy, as stated in the Global Strategy of Plant Conservation (UNEP 2002), and is frequently employed by a number of organizations known for biodiversity conservation (Sarasan et al. 2006). Plant tissue culture technique has been reported as an effective tool to conserve many plant species, especially of tropical origin (Engelmann 1991). Moreover, *in vitro* conservation is a better way for storage of disease-free elite clones and for exchange of genetic resources (Withers 1991; Crouch et al. 1998; Engelmann 1998; Theilade and Petri 2003). There are three categories of *in vitro* conservation *viz.*, short-term (*in vitro* cultures), mid-term (slow growing cultures) and long-term (cryopreservation). For the short- and mid-term conservations, various techniques have been developed which not only results slow-growth of the cultures but also prolong the time-interval between two subcultures (Cha-um and Kirdmanee 2007).

However, the main difficulties associated with long-term maintenance of *in vitro* cultures are that the procedure is a bit problematic and expensive as it involves huge amount of resources and labour (Rao 2004; Capuana and Lonardo 2013). *In vitro* slow-growth, therefore, represents a possible solution for mid- to long-term storage of plant materials in limited space and at reduced costs. Moreover, slow-growth procedures allow clonal plant conservation for several months to year (depending upon the species) under aseptic conditions, requiring infrequent subculturating (Cha-um and Kirdmanee 2007).

Further, *in vitro* tools involving slow-growth techniques have paved the way to conserve species *ex situ* and have been employed successfully with a number of plant species (Tyagi et al. 2009; Lata et al. 2012; Peng et al. 2015; Silva et al. 2016; Ozudogru et al. 2017), including vegetatively propagated varieties (Negash et al. 2001; Bekheet 2002; Thakur et al. 2015). The slow-growth techniques are widely used for conservation of plant diversity because in these various genotypes can be effectively preserved without inducing any loss of viability and also in the form of disease-free planting stock, under controlled environment. Slow-growth techniques are primarily based on conditions that allow minimal growth of cells, tissues or organs, and can be achieved by maintaining reduced storage temperature or by addition of osmotic regulators and growth retardants into the culture medium. In normal course, the most widely applied procedures to minimize growth are manipulation in the culture medium, reduction in the storage temperature or both (Engelmann 1997).
Cryopreservation is one of the storage methods of plant genetic resources at ultra-low temperature (-196°C), usually in liquid nitrogen (LN; Benson 2008; Chen et al. 2015). It is a preservation method that enables plant genetic resources to be conserved safely for long-term, and in cost-effective manner. At cryogenic temperature (below -130°C), approximately all sort of metabolic activities and division of cells become ceased, hence samples can theoretically be preserved for hundreds of years in limited space, in comparison to materials grown in vitro or in the field (Benelli et al. 2013). However, tropical and sub-tropical plant species are sensitive to both desiccation and low temperature damage, and quickly loss viability (Gonzalez-Arnao et al. 2008). That is why, effective cryopreservation protocols for a limited varieties of tropical origin are till date available as these species are quite prone towards low temperature (<10°C) which results in lower survival percentage or death after cryo-storage (Angeli et al. 2003; Li et al. 2004; Gusta et al. 2005).

Thus, for successful cryopreservation, it is essential to avoid intracellular freezing of water, and to induce vitrification of the cells during cooling in ultra-low temperature. In addition, the cryopreservation method should be a simple protocol so that anyone can be able to use it easily. Since 1970’s, cryopreservation techniques have been worked out for a number of species, but mostly for the temperate taxon. As a result, various cryopreservation procedures have been developed (for example, vitrification, dehydration, and droplet vitrification). With the development of these cryopreservation methods, tissues of some of the tropical plant species, which have been conventionally thought to be not cryopreserved, can also be preserved successfully under LN (Bajaj 1995; Towill and Bajaj 2002; Benson 2008).

Now-a-days, cryopreservation has become preferred option for making repository of clonally propagated plant germplasms and also to ensure the safe and cost efficient long-term conservation (Keller et al. 2008; Nukari et al. 2009). It is now applied to a diverse range of species, including Musa sp. (Panis et al. 2010), Rubus idaeus, Humulus lupulus (Zamecnik et al. 2007; Haggman and Uosukainen 2010), Solanum tuberosum (Keller 2007; Zamecnik et al. 2007; Gonzalez-Arnao et al. 2008; Nukari et al. 2009), Allium sativum (Keller 2007; Kim et al. 2012), Mentha spicata (Keller 2007), Malus domestica (Forsline et al. 1998; Lambardi et al. 2011; Hofer 2015), Pyrus communis, Prunus sp. (Zamecnik et al. 2007), Dioscorea sp. (Keller et al. 2008), Musa sp., Citrus sp., S. tuberosum and Saccharum officinarum (NBPGR 2015), etc.
1.3 *Plant Secondary Metabolite and Elicitation Strategy*

The production of secondary metabolites following the commercial cultivation of plants is an important agronomic and industrial objective. The chemically synthesized metabolites in most of the cases have not been economically feasible. Therefore, the promising substitute is *in vitro* culture, which represents a potential source of bioactive molecules.

Plants synthesize a range of miscellaneous structurally complex and low molecular weight chemical compounds, generally known as secondary metabolites. These metabolites have an extremely important role in plants interaction with surrounding environment in terms of their existence and sustenance (Pichersky and Gang 2000; Moore et al. 2014). Higher plants make their own self-defence strategies, either structural or chemical, for survival. As a component of chemical defence strategy, plants synthesize a number of secondary metabolites to meet out their requirements. According to an estimate, plants produce more than one lakh type of secondary metabolites, while this number may exceed over five lakh once they are structurally characterized (Hadacek 2002).

A number of metabolites are yet to be characterized fully for their usage in health care and pharmaceutical bio-prospecting (Lee 2010; Soejarto et al. 2012; Elkington 2014; Pan 2014). On the other hand, a number of such chemical compounds are in use currently for medicine formulations, and serves as the chief source of several important bioactive pharmacophores (Dias et al. 2012; Pan 2014; Raomai et al. 2015). At present, one-fourth of all the prescribed pharmaceuticals, across the world, contain secondary metabolites that are directly, indirectly or semi-synthetically derived from plants exclusively (Song et al. 2014).

As these secondary metabolites are produced in small quantities, there is an urgent need to search such a system which can be able to produce these metabolites at commercial scale. During *in vitro* culture of plants or its parts, addition of some of the potential signal compounds or elicitors have been reported to enhance the production of secondary metabolites. These elicitors are able to cross talk with the receptors which are present in the plasma membranes of plant cells by stimulating the defence responses (Gorelick and Bernstein 2014). Moreover, elicitors have also shown to regulate different biochemical control points, and trigger expressions of key genes along with transcriptional factors.
Hence, it is now well established that the potential signal compounds have the ability to control a range of cellular activities at the biochemical and molecular level (Zhao et al. 2005; Baenas et al. 2014). Therefore, despite of immense advancements in the area of synthetic chemistry, people still depend largely upon biological sources for a number of secondary metabolites, including pharmaceuticals.

1.4 Chlorophytum borivilianum - Studied Species

*Chlorophytum borivilianum* is one of the commercially popular herbs known as ‘Safed Musli’ in India, commonly (Maiti and Geetha 2005). It occupies a significant place among Ayurvedic crude drugs hence gaining higher trade value. It also has obliged for the crops genetic improvement programs (Kumar et al. 2008). *Chlorohytum borivilianum* is an important member of the family Liliaceae and genus *Chlorophytum* Ker Gawl, which in recent years has fascinated the scientific community owing to the presence of bioactive constituents which are saponins and sapogenins, particularly (Kaushik 2005; Acharya et al. 2008, 2009; Thakur et al. 2009). Globally the genus *Chlorophytum* is represented by more than 200 species and distributed particularly in tropical and subtropical countries (Govaerts et al. 2012). In India, genus *Chlorphytum* is represented by about 17 species, of which 15 are reported to be found in the peninsular region, and among which 9 are endemic in this country only (Chandore et al. 2012), and one of them is *C. borivilianum*.

At the commercial scale, *C. borivilianum* is one of the heavily exploited species (Tandon and Shukla 1995). In India, this taxon was initially identified by Santapau and Fernandes (1954) and was documented to constitute the highest amounts of saponin and sapogenin, which ranged between 2 - 17% and 0.18%, respectively of its dry weight (DW), depending upon the genotype (Bordia et al. 1995). Because of its important bioactive constituent, it was exploited rigorously as a very good alternative of Viagra; a well-known drug to overcome sexual dysfunction (Kaushik 2005; Thakur et al. 2009). Looking to its massive commercial exploitation and declining population, Nayar and Sastry (1988) enlisted this species in Red Data Book of Indian Plants as “Rare Plant”, which was developed by the Botanical Survey of India, Kolkata (India), and more recently International Union for Conservation of Nature and Natural Resources (IUCN 2015) referred this species as “critically endangered”.

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High cost and poor availability of good planting materials are the major problems encountered by cultivators of *C. borivilianum* (Oudhia 2001). Therefore, for commercial cultivation, vegetative propagules of it were popularly used by the growers (Bordia et al. 1995; Kaur et al. 2009). Limited viability and vigour of its seeds are the major reasons behind use of vegetative propagules as a chief source of planting stock. Germination percentage of *C. borivilianum* seeds was documented to be quite less *i.e.* 8 - 13% only, also were not able to produce quality seedlings with desired growth potential (Bordia et al. 1995; Kaur et al. 2009). In order to overcome these challenges, various investigators developed micropropagation protocols for this species (Purohit et al. 1994; Dave et al. 2003; Rizvi et al. 2007). However, for conservation of germplasm of it, only single attempt has been made till date that too with a little success (Dave et al. 2004).

1.5 Objectives

Considering the commercial importance and unplanned over exploitation of *C. borivilianum*, the present study was aimed to deal with below listed objectives;

a) Developing suitable *in vitro* slow-growth storage protocols for micro-shoots of targeted critically endangered species.

b) Standardize protocol for cryopreservation of meristematic-tips for the long-term *ex situ* conservation of selected species.

c) This study also aimed to develop methods for enhancing the secondary metabolites of *C. borivilianum* through elicitation by potential signal compounds.