SEED GERMINATION AND MICROPROPAGATION STUDIES OF MAMMEA SURIGA KOSTERM

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ABSTRACT

Mammea suriga Kosterm. is a RET species distributed in Malnad region of the Western Ghats, which is over exploited for flowers, seeds and timbers, hence the population is declined. The study was conducted to evaluate the germination potency of the seeds in both in vivo and in vitro conditions to derive a regeneration protocol for the ex situ conservation. Among the pre-treated seeds, the decoated seeds pre-treated with 0.5 mg L^{-1} NAA for 24 h resulted in 86 % of germination. In in vitro condition, culture of decoated seeds on MS medium supplemented with 0.5 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ resulted 91.67 % of germination. Micropropagation studies showed that the interaction of 2-3 mg L^{-1} BAP and 0.2 mg L^{-1} TDZ induced multiple shoots from cotyledonary and hypocotyl explants. Mass of callus induced from the leaf explants at the concentration of 1 mg L^{-1} 2,4-D and 3 mg L^{-1} BAP. Later on the photosynthetic callus, organise in to shoot buds at the concentration of 3 mg L^{-1} BAP and 0.2 mg L^{-1} TDZ. The excised micro shoots showed rhizogenesis on the media fortified with 1.5 mg L^{-1} IBA. The regenerants derived from both direct and indirect organogenesis exhibited similar morphological features.

Keywords: Mammea suriga, seed germination, micropropagation

INTRODUCTION

Plants are valuable sources of medicinal and many other pharmaceutical products. The conventional methods of propagation take a long time for multiplication because of a low rate of fruit set, along with poor germination. The plants used in the phyto-pharmaceutical preparations are obtained mainly from the natural growing areas. With the increase in the demand for the drugs, the plants are being overexploited, threatening the survival of many rare species. Also, many medicinal plant species are disappearing at an alarming rate due to depletion of habitat, rapid agricultural and urban development, uncontrolled deforestation, and indiscriminate collection. Advanced biotechnological methods of cultivating plant cells and tissues should provide new means for conserving medicinal plants. The use of controlled environments in these techniques can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins (Pradeepa, 2016; Venkatesh, 2017; Santhosh Kumar, 2017). Therefore, standardisation of micropropagation protocol and ex situ conservation of threatened and endangered medicinal plant species is of utmost importance.

Mammea suriga is found in evergreen forests of western India from Khandala southwards to Malabar and Coimbatore (Pulliaah, 2006), cultivated in Assam, West Bengal, Orissa and Uttar Pradesh (pillkula.com/botanical_list/botanical_name_m/mammea_suriga.html). It is also found in Ceylon and West Indies (Chopra 1956). Mammea suriga Kosterm. belongs to the family Calophyllaceae. It is a medium-sized tree bearing fragrant white flowers. It is commonly called as Indian rose chestnut or Ceylon ironwood and vernacularly called as Punnag (Sanskrit), Surangi (Hindi), Suragi (Kannada) etc. (http://en.wikipedia.org/wiki/Mammea_suriga). Leaves are simple, opposite, thickly coriaceous, dark green, base rounded, midrib stout prominent, veins few, indistinct, very slender, and beautiful. Androdiocoeous tree species-male flowers on one plant and either female or bisexual flowers on other plants. The flowers cauliflorous- born on old stem during hot season, (http://indibiodiversity.org/species/show/14963), sprouts as beautifully rounded buds, which bloom into four-petaled white flowers. The flowers are extremely fragrant, chiropterophilous, and its powerful scent can hit the nostrils even from a long distance. Fruit ripens during rainy season, and it is obliquely ovoid, 1-seeded. It is also reported that some of the fruits of this species have double seeds. Moreover, they have quantified rate of double seeds among different individuals within a population. (flowersofindia.net/catalog/slides/Surangi.html). Due to handsome foliage and sweetly scented flowers, this plant is used in avenue planting program. Fresh flowers of the tree are used for worshipping in temples as well as in personal adornment. In the local floral market per kg of flower buds costs 150-200 Rupees. The dry flowers maintain fragrance for long time; therefore, flowers are used in perfumery industries. The silky red dye extracted from dried flower is used in textile industries. The flower bud has medicinal properties and used as an astringent as well as in dyspepsia. Hence, this species is considered as multi-purpose tree species of Western Ghats (Smita, 2013). In traditional medicine, flower buds are mainly used for skin eruption, itching, small tumours, blood and heart troubles, sore throat, cough, vomiting, dysentery, bleeding piles, for fevers, foul breath. It is also used as mild stimulant, adulterants, carminative and astringent properties and is used in dyspepsia (Pullaiah, 2006). The root bark showed antimalarial activity (Valsaraj et al., 1995) and used to cure leprosy, eczema and other skin diseases (Prusti and Behera, 2007). This species is very sparsely found in the forests of North Canara of the Western Ghats and as per CAMP (2001), IUCN RED List (2007) and ADMA RED list, Mammea suriga Kosterm, is reported as a threatened tree species endemic to the Western Ghats (Ankur and Vasudeva, 2003). In this investigation, we report the protocol for the enhancement of germination potency of the seeds in both in vivo and in vitro conditions to derive a regeneration protocol for the ex-situ conservation of this threatened species.
MATERIALS AND METHODS

Seed germination studies

*Mammea suriga* immature seeds were collected from a healthily grown tee in May. For *in vivo* germination studies before use, unsterilized seeds were subjected to various pre-treatments, viz. soaking in water for 48 h, 0.1 % KNO₃, 0.5 mg L⁻¹ NAA treatment for 24 h, decoated seeds, decocked seeds pre-treated with 0.5 mg L⁻¹ NAA for 24 h. The seeds were washed with tap water to remove traces of chemicals and were sown directly in the soil at a depth of 3 cm in a tray. Watering was done every day. Germination of the seeds was tested in room temperature; seeds were sown in replications containing 100 seeds each. The observations on germination were recorded. For *in vitro* germination studies the seeds were sterilised prior to use with running tap water followed by, tween 20 and with 70 % ethanol, finally sterilised with 1 % mercuric chloride and washed three times with sterile water. Seed coat was removed and inoculated in MS medium supplemented with 2 to 5 mg L⁻¹ of BAP and 0.1 to 0.5 mg L⁻¹ of TDZ for germination. Cultures were incubated under fluorescent lights at 25° C, the humidity was maintained at 85 % under a photoperiod of 16 h of light (25 µmol s⁻¹ m⁻²), and eight-hour dark, and the cultures were monitored daily. In all the above experiments, after observing callus induction, multiplication of callus and shoot proliferation, the cultures were allowed to grow further. Subsequently, multiple shoots formed were dissected into individuals and sub cultured on to the same MS media. Shoots with at least 2-4 well-developed leaves were transferred for rooting. After 20-40 days of incubation, induction of shoot organogenesis in terms of a number of shoots per explants was recorded and statistically analysed using eZANOVA 3.0 and represented as mean ± SD.

Rooting and acclimatisation

Healthy shoots with minimum 4-6 leaves were transferred to MS medium with 2 mg L⁻¹ IBA for a root formation. Root formation was evaluated after 15 days. Further plantlets were allowed to grow for another 14-15 days. Well-developed plantlets were transferred to seed trays containing a mixture of vermiculite and sand in equal ratio and humidity is maintained for further growth of plantlets by covering the plantlets with a polythene bag. Further, the well-grown plantlets were transferred to pots with gardening soil for secondary hardening.

RESULTS

Micropropagation of *Mammea suriga*

**In vivo seed germination**

Initially, experiment was conducted to evaluate the germination potency of *Mammea suriga* seeds in the *in vivo* condition. Seeds are soaked in water for 48 h and then sown in sterilised soil bed. Commencement of seed germination was noticed after 42 days, and only 20 % of the seeds were germinated. In pre-treatment with 0.1 % KNO₃, and 0.5 mg L⁻¹ NAA for 24 h only 38 % and 46 % respectively, of intact seeds were germinated. Germination noticed after 30 days. Whereas, the percentage of germination was enhanced to 72 % in the decoated seeds and seed germination was observed after 30 days. The decoated seeds pre-treated with 0.5 mg L⁻¹ NAA for 24 h resulted in 86 % of germination, and the percentage data is shown in figure 2.

![Figure 2 Percentage of seed germination in vivo condition.](image)

**Legend:** The X-axis represents treatments, and the Y-axis represents the percent of germination of seeds, the bars represent Mean ± SE.
**In vitro seed germination**

Commencement of seed germination was started after 15 days of incubation, and the percentage of seed germination was enhanced to 91.67 % after 30 days of incubation (figure 3).

![In vitro seed germination graph](image)

**Figure 3** Seed germination in *in vitro* condition on MS media

Legend: The X-axis represents treatments and the Y-axis represents time (days) of germination of seeds, the bars represent Mean ± SE.

**Micropropagation studies**

**Direct organogenesis**

The *in vitro* shoot regeneration was noticed directly from the cotyledon and hypocotyl explants in the combinations of BAP (3 mg L⁻¹) and TDZ (0.2 mg L⁻¹) (Table 1) without undergoing callogenesis phase. In cotyledon explant culture, multiple shoot organogenesis was found to be the best at a concentration of 2 mg L⁻¹ BAP and 0.2 mg L⁻¹ TDZ. Before shoot organogenesis, growth and enlargement of cotyledonal explant were noticed. After three weeks of culture, the explant became photosynthetic, and organogenesis of multiple shoots was noticed from the cotyledonal node of the explant. In hypocotyl, explant culture organogenesis of shoots noticed from the nodes and the excised end of the explants. At the concentration of 3 mg L⁻¹ BAP and 0.2 mg L⁻¹ TDZ, 1.67±0.58 shoots were organised from the hypocotyl explants. Within a week of incubation, elongation of the excised explants were noticed. Before initiation of the shoot, bud’s nodal region became swollen and sprouting of shoot buds were noticed after 4 weeks of culture. The organised shoots grew up with photosynthetic leaf primordia.

After 6 weeks of culture, the well-grown micro shoots with 2-3 leaves were excised aseptically and sub cultured on to rooting media supplemented with 0.5 to 4 mg L⁻¹ IBA. At a concentration of 1.5 mg L⁻¹ IBA rhizogenesis noticed from the basal node of the micro shoots. The well-rooted plants were isolated, media attached to which was cleaned and transferred to pots containing a mixture of vermiculite and sand in equal proportion. The plantlets were initially hardened in the culture room by covering with a polythene bag to maintain the humidity; later secondary hardening was carried out in the greenhouse condition (figure 4).

**Table 1** Effect of interaction of BAP and TDZ on callogenesis from the cotyledon and hypocotyl explants

| Growth regulators (mg L⁻¹) | TDZ | No. of shoot buds |
|---------------------------|-----|------------------|
| **Cotyledon explants**    |     |                  |
| 1                         | 0.1 | 0                |
| 2                         | 0.5 | 0                |
| 3                         | 0.2 | 2.67±0.58        |
| 4                         | 0.2 | 1.33±0.58        |
| **Hypocotyl explants**    |     |                  |
| 1                         | 0.1 | 0                |
| 2                         | 0.2 | 1.67±0.58        |
| 3                         | 0.2 | 1.0±0.0          |
| 4                         | 0.2 | 0                |

Legend: The values consist of Mean/SD of three independent experiments.

**Table 2** Effect of growth regulators on leaf explant culture

| Growth regulators (mg L⁻¹) | BAP | No. of shoot buds/callus |
|---------------------------|-----|-------------------------|
| 1                         | 0   | 0                      |
| 0.1                       | 0.1 | 0                      |
| 0.2                       | 0.2 | 0                      |
| 0.5                       | 0.5 | 0                      |
| 1.0                       | 1.0 | 0                      |
| 1.5                       | 1.5 | 0                      |
| 2                         | 2   | 0                      |
| 2.5                       | 2.5 | 0                      |
| 3                         | 3   | 1.67±0.58              |
| 4                         | 4   | 1.0±0.0                |

Legend: The values consist of Mean/SD of three independent experiments.

**Indirect organogenesis**

**Leaf explant culture**

The leaf explant of *M. suriga* inoculated onto MS medium augmented with a range of 0.5 to 2.0 mg L⁻¹ 2,4-D, and 1 to 5.0 mg L⁻¹ BAP showed callogenesis has shown in figure 5 and table 2. Callus was first initiated from the midvein of the excised leaf disc. Later the whitish mass of callus was spread over the excised end and dorsal surface of the leaf explant. The primary callus was whitish hard nodular compact mass. Luxuriant callus proliferation was noticed after sub culturing on the same medium. On shoot, differentiating media fortified with 2 to 4 mg L⁻¹ BAP and 0.1 to 0.5 TDZ. The whitish mass of callus turned to brown and became hard and nodular. The initiation and differentiation of shoot buds from the leaf callus mass were noticed at a concentration of 3 mg L⁻¹ BAP and 0.2 mg L⁻¹ TDZ. As compared to juvenile explant cultures, the caulogenic efficacy was found to be less in leaf explant culture. Only one or two shoots grew up from each mass of callus. The well-grown leaf callus regenerated micro shoots were transferred to rooting media supplemented with 1.5 mg L⁻¹ IBA. As observed in the case of cotyledonal culture, the leaf calli regenerated shoots were also showed the organogenesis of the roots from the basal nodes. However, the frequency of rhizogenesis was found to be less. The well-grown leaf calli regenerated plants were subjected to primary hardening, secondary hardening and transferred to pots containing the garden soil. The morphology of the 6 months old leaf calli regenerated plant was found to be similar to the plants of direct organogenesis from cotyledonal and hypocotyl explants.
supplemented with NAA and TDZ. The decoated seeds grown in in vitro condition have shown increased percent of germination, which may be because of several factors such as, supplemented nutrients, the combination of the auxin and cytokinin, less exposure to the various climatic factors as well as the infections and other barriers, removal of seed coats may helpful in responding to the in vitro growth condition quickly and rapidly. And maybe accredited to the fact that at a supplemented concentration of plant growth regulators, increased the stimulation of the expression of enzymes, affecting both the physiological and metabolic activities within the seeds thereby, promoting embryo growth and reducing the physical restraint imposed by the endosperm or seed tests making way for the protrusion of the radicle. Also, effective in overcoming dormancy, thereby causing rapid germination of seeds (Singh and Murthy, 1987; Jan and Shekhawat, 2011).

Recently Shikhasmita et al., 2016, Chary et al., 2017, and Ajith et al. 2018 have also investigated the breaking of seed dormancy of other various endemic, endangered medicinal plants and succeeded in breaking of seed dormancy by employing various treatments. In M. suriga Rajesh et al., 2011 have reported the germination in intact seeds in relation to the seed size and also the germination of seed on treatment with cow dung slurry.

Micropropagation studies

Micropropagation is the method of vegetative growth and reproduction plants from, tissues or seeds. It is carried out in aseptic and favourable conditions on growth media, using various plant tissue culture techniques (Fowler et al., 1993; Bhogvari and Raardon, 1996; Zhou and Wu, 2006). Tissue culture is based on the theory of totipotency; the ability of plant cells and tissues to develop into a whole new plant (Fowler et al., 1993). Gottlieb Haberlandt (1854-1945), a German botanist is considered as the father of plant tissue culture, was the first to separate and culture plant cells on Knop's salt solution in 1898 (Krikorian and Bergmann, 1969). Micropropagation has the potential to provide a multiplication rate of selected tree genotypes. In conservative cultivation, several plants do not sprout, flower and produce seeds under certain climatic situations or have extensive periods of growth and multiplication. Micropropagation insures a good consistent supply of medicinal plants, using minimum space and time (Prakash and Van staden, 2007). From the last few decades, there has been a good increase in research on the medicinal plant. A number of new medicines have been discovered and advancements in production technology to harvest pharmaceutically important metabolites.

About 40 % of compounds used in the pharmaceutical industry are directly or indirectly derived from plants (Stafford, 1986) because the chemical synthesis of such compounds is either not possible and economically not viable (Oksman and Inzé, 2004). Therefore, a huge number of plant species (especially medicinal) are under threat of extinction because of their over-exploitation (Vines, 2004; Rout et al., 2000; Edwards, 2004).

Plant tissue culture studies were carried out for the preservation of medicinal plant resources and efficient production of pharmaceutically important secondary metabolites. Micropropagation methods for M. suriga were established, and these methods enabled much more efficient proliferation of plants than the conventional approaches using seedling or layering. In our study, TDZ was found to be more effective in the induction of multiple shoots as compared to other cytokinins. The synergistic effects of TDZ with other cytokinin/auxin was found more effective than using alone.

Direct organogenesis

The production of buds or shoots directly from the plant tissue without intervening callus phase is termed as direct organogenesis. Plants have been proliferated by direct organogenesis for better reproduction rates, production of transgenic plants, clonal propagation, and most importantly for the conservation of genetic material of many threatened, endemic and vulnerable medicinal plants. The axillary bud induction or multiple shoot initiation methods are the most common means of micropropagation since it ensures the production of even planting material without genomic variation. Axillary shoots are formed directly from explants, and the chances of the organised shoot meristem undergoing mutation are comparatively low. This technique is referred to as multiple bud induction. Various economically important plants have been propagated using this method.

The in vitro shoot regeneration was achieved directly by cultivating cotyledon and hypocotyl explants on MS medium supplemented with combinations of BAP and TDZ without undergoing callusogenic phase. In vitro regeneration of plants is influenced by many factors such as environment around cultures, media composition, and source of explant, plant growth hormones and genotype (Zhang, 1998; Bano, 2010; Jana and Shekhawat, 2011; Dhir and Shekhawat, 2014). In our study, high-frequency direct regeneration from cotyledonary explant was achieved at the concentrations of 2 mg L–1 BAP and 0.2 mg L–1 TDZ. In hypocotyl explant, culture best callusogenic response was observed at the concentration of 3 mg L–1 BAP and 0.2 mg L–1 TDZ, and the organogenesis of shoots was achieved directly from the nodes and apical region of the hypocotyl explants.
Many researchers have also reported the regeneration of plantlets from cotedledon explants viz, Sinapis alba (Jain, 1989), P. coccineus L. (Angelini and Allayene, 1989), Fagara ass-fouetida L. (Zaire et al, 2010), Millettia pinnata (L.) (Nagar et al, 2015), A. melanocarpa, P. communis, and A. mitchukhini (Jonathah et al, 2018), have reported the regeneration of plantlets from cotedledonary explants. Similarly, hypocotyl explants were also used for the organogenesis of shoots, viz. Brassica carinata A. Br. (Yang et al, 1991), Arabidopsis thaliana (Gendreau et al, 1997), Brassica napus L. (Tang et al, 2011), Brassica oleracea cv. (Gambhir et al, 2017), Zygophyllum potaninii (Bayarmaa et al, 2018), Solanum quitoense (Gutiérrez et al, 2019).

**Indirect organogenesis**

Development of plantlets directly through the callus is called indirect organogenesis. Genetic purity of plantlets using this method ensures mass multiplication and conservation of species and its unique traits. Since the age of explant is of high paramount importance in tissue culture, the younger explants are more responsive than, the older one. Furthermore, sterilisation of younger tissue is easier than the older tissue. Our study, for the first time, indicated the candidature of the leaf explant of *M. suriga* inoculated onto MS medium supplemented with 2,4-D and BAP. The mass of calli turned from whitish to brown, showed differentiation of shoot buds. The well-grown plantlets after rooting stage maintained under culture condition are processed further for primary and secondary hardening. Similarly, regeneration of plantlets from leaf explants of *Ficus religiosa* (Hesami et al, 2018), Solanum quitoense (Gutiérrez et al, 2019), has been achieved and reported.

The majority of woody plants and some herbaceous species cause browning of the explants and callus, followed by necrosis of cells and causes death (Zhang et al., 1998). The browning of the medium is due to the release of phenol by the explants, which gets oxidised, and this oxidation product could be phytotoxic. Therefore, there needs to be a scrutinised investigation before incubating explants in the culture medium (Ko et al, 2009). The degree of browning is different from species to species and depends on the age of the tissue (old tissues show more browning than the younger one), the season of culture initiation (more in winters and autumn) and composition of the medium (Sienza et al, 2010). Another major drawback is the presence of endophytic contaminants in the woody species, which will interrupt the regeneration process of the explants in *in vitro* condition.

**CONCLUSION**

The role of medicinal plants in pharmacoeconomics and other industries is strengthening India’s economy and cannot be exclusively depend on natural botanical sources. The present study was so based & aimed at the derivation of *in vitro* regeneration protocol for *M. suriga*. In the present study, we have emphasised the *in vitro* regeneration protocol to conserve *M. suriga*, which is considered as RET species. This plant is a novel source of phytochemical and the regeneration protocol to conserve *M. suriga* has been achieved and reported. This plant is a novel source of phytochemical and the regeneration protocol to conserve *M. suriga* has been achieved and reported.

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