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In vitro seed germination and Embryo Culture in *Nothapodytes foetida* (Wight) Sleumer

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ABSTRACT

In vitro seed germination and embryo culture have been achieved in *Nothapodytes foetida,* this plant is known for its rich source of anticancer drug i. e., Camptothecin. In present study both normal and decoated seeds were subjected to different treatments viz., H2O, GA_3, H2O2, H2SO4, chlorine water and mechanical scarification, further these were germinated on water agar medium (WA), filter paper bridge (FB), half strength MS (HMS) and full strength MS (FMS) medium. The highest percentage (69%) of germination was achieved from decoated seeds treated with 10mg/L GA_3 and germinated on Filter Paper Bridge. And for embryo culture mature embryos were inoculated on MS medium containing various combination and concentrations of cytokinins (BAP, Kn and TDZ) and auxin (IAA and NAA) for rapid conversion into a plantlet. Among the different combinations of growth regulators; highest frequency (100%) of plantlet conversion was obtained on MS medium containing Kn (1.0mg/L) and NAA (0.2mg/L).

1. INTRODUCTION

Nothapodytes foetida (Wight) Sleumer (formerly Known as *Mappia foetida* and *N. nimmoniana*) belongs to family Icacinaceae. It is a small tree, naturally distributed in many parts of the Western Ghats and a rich source of the potent cytotoxic quinoline alkaloids camptothecin and 9-methoxycamptothecin (Fulzele *et al.*, 2001) and its accumulation was reported in many parts of plant (Padmanabha *et al.,* 2006). Camptothecin was isolated for the first time from *Camptotheca acuminata* of Nyssaceae (wall *et al.*, 1966), and was further detected in *Nothapodytes foetida* (Govindachari and Vishwanathan 1972). CPT is a cytotoxic quinolone alkaloid and is used as an antitumor drug in the treatment of head, colon, breast and bladder cancers. It also inhibits the replication of retroviruses such as human immune deficiency virus (HIV) (Pantazis *et al.*, 1996). Due to exploitation of this resource and extremely high pressure, the species has been declared as endangered (Ved, 1997). In the recent years, several independent groups have addressed the need to conserve this species and to explore the possibility of identifying high-yielding individuals and/or populations for the development of *in vitro* production systems (Ciddi *et al.,* 2000 and Roja *et al.*, 1994). Camptothecin is the third most promising alkaloid of the twenty-first century and is produced in various tissues of *N. nimmoniana* at variable yield quantity with highest accumulation in stem and bark of the tree (Panneerselvam *et al.*, 2004; Lorence and Nessler 2004; Padmanabha *et al.,* 2006; Suhas *et al.*, 2007; Namdeo and Sharma 2012).

In tree species seed germination is difficult task due to hard seed coat and dormant embryos (Jaiswal and Chaudhary 2005) and they often fail to germinate even under favorable conditions like optimum availability of moisture, oxygen and soil conditions (Urgenc and Cepel 2001). Pretreatments to the seeds like mechanical scarifiation, soaking in cold or hot water, acid scarifiation with sulphuric or hydrochloric acid (Bedell 1998) and pre-soaking of seeds in growth regulator like gibberellic acid (GA_3) and indoleacetic acid (IAA) (Chauhan *et al* 2009) are known to overcome dormancy of seeds and can also enhance seed germination percentage. Embryo culture is also plays an important role in *in vitro* propagation of endangered forest tree species to overcome

physical and biotic interferences. Hence in the present study an attempt is made to develop a protocol for *in vitro* seed germination, Embryo culture and plantlet conversion in *N foetida.*

Materials and Methods:

Preparation of explants

The fruits were collected from forest area of Mahabaleshwar (Maharashtra). Seed coat was removed manually the normal seed and de-coated seeds were washed under running tap water followed by treating with Tween 20 and Bavistin for 15min, then soaked in 4% Sodium Hypochlorite for 10min with occasional stirring and washing with deionized water followed by soaking in 70% ethyl alcohol then these seeds were washed with distilled water. Finally seeds were surface sterilized in 0.1% $HgCl_2$ for 2-3 min in laminar airflow hood and washed with deionized water 3-4 times to remove the traces of $HgCl_2$. Further both normal and decoated sterilized seeds were subjected to different treatments as enumerated below.

Methodologies employed for seed germination:

A. **Water treatment:** Normal and decoated seeds were soaked in distilled water for 24hrs and 48hrs in dark
B. **GA3 treatment**: Seeds weresoaked for 24hrs in 10 and 20mg/l GA3 in dark condition at room temperature.
C. **Acid treatment or scarification:** Normal and decoatedseeds were treated with 10 and 20% sulphuric acid for 10 and 20min.
D. **H_2O_2 treatment:** Seeds with and without seed coat were treated with 1, 2, 3 and 4% hydrogen peroxide (H_2O_2) for 4hrs.
E. **Chlorine water treatment:** Seeds with and without seed coat were treated with chlorine water for 10 and 20min.
   Seeds were inoculated on either petriplates containing moist sterilized filer paper, full strength MS medium (FMS), half strength MS medium (HMS), filter paper bridge (FPB) or water agar medium (WA).
F. **Mechanical Scarification:** Both normal and decoated seeds were scarified and made a minute opening at the opposite end of the embryo attached to the seed to imbibe more amount of water and nutrient molecules.

Embryo culture and culture conditions

Mature embryos are dissected from the seed and used as explant. They were then aseptically inoculated on Murashige Skoog's medium (MS) supplemented with various concentrations and combinations of auxins (NAA and IAA) and cytokinins (BAP Kn and TDZ). The medium was gelled with 0.8% Agar (Himedia) 50 explants per treatment and three replications per study were maintained. All the cultures were incubated in light and dark (16x8 h) conditions of light providing a quantum flux density of $30 lmol S^{-1} m^{-2}$ provided by cool- white- fluorescent bulbs at $25 \pm 2^0 C$.

Result

Seed germination:

Normal and decoated seeds when subjected to all type of treatments as mentioned in materials and methods failed to germinate when grown in petriplates containing filter paper moistened with sterilized distilled water; however the response of decoated seeds after subjecting to water soaking (24 hrs) differed depending on the type of germination medium. The percent germination observed was 14% when placed on full strength MS (FMS) and 17% on half strength MS agar medium (HMS) and 25% when they were placed on Filter Paper Bridge (FB) in culture tubes containing MS Liquid medium. However normal seeds soaked in distilled water for 48hrs and placed on different germination medium as referred to above showed delayed germination (8days) when placed on FMS (9%), HMS (11%) and FB (17%).

GA_3 treatment at 10mg/L of decoated seeds showed early (4days) and increased percent of germination; it was 69% on FB, 48% in HMS, 43% in FMS and plain agar medium (WA) 41%. Seeds soaked in 20mg/L GA_3 showed delayed and reduced percentage of germination, when grown

under similar conditions mentioned above. The normal seeds showed delayed and reduced percent germination than the decoated seeds when subjected to GA_3treatment and grown under similar conditions as mentioned above.

Both normal and decoated seeds were subjected to 10 and 20% H_2SO_4 treatment for 10 minutes. H_2SO_4 treatment proved lethal to decoated seeds as they failed to germinate when treated with both 10 and 20% H_2SO_4. However normal seeds treated with 10% H_2SO_4 showed 25, 21, 20 and 10% germination when placed on FPB, HMS, WA and FMS respectively. 20% H_2SO_4 treatment showed a decrease in the percent germination to 15, 14, 12 and 6% when germinated on similar media as mentioned above.

Both normal and decoated seeds were superficially scratched with the help of sand paper to make an opening at the opposite end of the embryo. Decoated seeds germinated early (5days) when compared to normal seeds (12days) on FB with a frequency of 29% and 19% respectively. While germination frequency of decoated seeds on HMS, FMS and WA was 21%, 17% and 12 respectively. Normal seeds had shown least germination on HMS (16%) and FMS (15%) and very poor on WA medium (6%).

Seeds (both type) treated with different concentrations of H_2O_2 (1-4%) for 4hrs. Among the different concentrations tested 3% H_2O_2 found superior over the other concentrations. After 3% H2O2 treatment decoated seeds showed better germination rate i.e., 37, 21, and 14% when placed on FB, HMS and FMS respectively, however normal seeds shown very poor germination on all type of inoculation media and no germination was recorded with both type of seeds on WA medium.

Both type of seeds treated with chlorine water for different time intervals (10 and 20min). Normal seeds failed to germinate on all type of inoculation media. However decoated seeds shown germination only on FB with very less frequency i.e., 9% and took 15 days to initiate germination.

Among the different types of treatments tested for germination decoated seeds treated with GA_3 (10mg/l) for 24h was found more suitable for *in vitro* germination than other treatments and among the different types of inoculation medium maximum percentage of germination was recorded on Filter paper bridge. In the present study 10mg/l GA_3 treated decoated seeds when inoculated in culture tubes containing Filter Bridge responded early and shown maximum germination with 69% frequency (Fig. 1).

Embryo culture:

Zygotic mature embryos of *N. foetida* were cultured on MS medium containing various concentrations and combinations of growth regulators. *In vitro* zygotic embryo germination started after 7-8 days of inoculation (**fig. 2. a**). The first visible sign occurred when the embryo changed color from white to yellowish. After 7-8 days of inoculation, radical elongation started **(Fig 2. b)** and feathery cotyledons changed from yellowish to green in color. Shoot emergence was observed after 10 days of inoculation **(Fig 2. c and d)** and when maintained up to 45 days well developed seedling emerged **(Fig 2. e and f)**. Embryos inoculated on MS medium augmented with cytokinins BAP and Kn (1-2mg/l) and TDZ (0.1- 0.2mg/l) or auxins like IAA or NAA (0.5-2.0mg/l) alone, failed to germinate and continued incubation lead to death of the explant. However combinations of cytokinins (BAP, Kn or TDZ) with auxins (IAA or NAA) showed beneficial results in germination of embryos. Rapid and high frequency of plantlet conversion was obtained on the medium containing 1.0mg/l Kn + 0.2 mg/l NAA (100%). Increase in the concentration of Kn to (1.5mg/l) and NAA (0.5mg/l) resulted in reduced frequency (66%) and seedling with drastically shortened roots and stem (fig--). However further increase in the concentration of NAA resulted in the callus formation at the transition zone of embryo and increasing the Kn concentration failed to convert into a complete plantlet. Where IAA was failed to respond at all concentrations and combinations along with cytokinins tested. Among the three cytokinins tested Kn was found superior for embryo germination where TDZ and BAP used alone or with NAA failed to produce plantlets at all concentrations. MS medium supplemented with 0.2mg/l NAA and 1.0mg/l Kn proved best combination for embryo germination both in terms of frequency and germination percent vigor (100%).

Discussion:

Seed germination:

Seed viability indicates the capability of seeds to germinate and produce normal seedlings under suitable germination conditions (Copeland and McDonald 2001). It has been known that three factors; temperature, seed moisture content and oxygen pressure are most important for viability and longevity of seeds in storage. Seed germination involves the protrusion of embryonic axis from the seed to resume plant growth (Finkelstein *et al.,* 2008; Park *et al.,* 2011). However, many seeds exhibit dormancy and fail to germinate even in favorable conditions. Seed germination is influenced by internal factors controlling dormancy, including phytohormones inducing dormancy (ABA), and seed coat factors (Baskin and Baskin 1998; Holdsworth *et al.,* 2008**;** Linkies and Leubner-Metzger, 2012).

The main purpose of this study is to optimize seed germination under *in vitro* conditions. The hard seed coat renders the seeds impermeable to water and oxygen needed for germination process (Baskin and Baskin, 1998). Different pretreatments are essential for effective germination and are species specific hence different trials have conducted to increase the germination potential. Mainly in *N. foetida* dormancy is due to physical presence of seed coat, physiological condition and presence of phenolic compounds at high extent in seed coat and in such a way results into inhibition of germination resulting in poor germination frequency *in vitro* (Sharma *et al.,* 2000; Khan 2013). Depending on the plant species and type of dormancy, various methods like scarification, stratification, removal of inhibitors and treatment with growth regulators are used to break dormancy (Baskin and Baskin 1998; Hidayati *et al.,* 2012). Soaking in water prior to sowing is also known to enhance germination percent and rate in different tree species (Bedell 1998). It is known that GA_3 enhances germination rate by breaking dormancy may be by activation of GA synthesis or by reducing the inhibitor level or both (Sharma *et al.,* 2000) and treatment of *Prunus mahaleb* seeds with gibberellic acid has been reported to overcome dormancy and ensure uniform germination (Cetinba and Koyunko 2006; Al-Absi 2010). In the present investigation also treatment with GA3 increased the percent of germination when inoculated on FB. In acid scarification method normal and decoated seeds are treated with 10% H_2SO_4while decoated seeds treated with acid failed to develop in all the germination types may be due to the death of nourishing tissues. However normal seeds upon acid treatment have responded well and shown good percentage of seed germination on FB. Many authors have reported beneficial effects of sulphuric acid in breaking seed coat dormancy in a number of leguminous plants (Mohammad and Amuse, 2003) and other plants which have hard seed coat (Hosseini *et al.,* 2013). The acid helps in removal of cuticle and softening of testa which facilitates water absorption and gaseous exchange (Govindachari and Viswanathan, 1972) however increase in acid concentration to 20% reduced the germination was recorded due to damage of embryo or killing the endosperm cells.

In the present investigation in all the germination methods tested decoated seeds inoculated on Filter Paper Bridge after soaking in GA_3 was found best method for germination. Filter papers can hold sufficient amount of water and dissipate water evenly all over the surface quickly and thus has great effect on rapid germination of seeds (Jaiswal and Chaudhary 2005).

Embryo culture:

Embryo culture is an important phase when there is poor embryo development or abortion of seeds and it repeatedly occur in early ripening genotypes where flesh matures before seed maturity thus causes poor germination of seeds and deprived embryo development. In many tree species like apricot and sweet cherry succeeded in obtaining seedling from embryo or to overcome problems of seed germination and in the present study we carried out germination of embryos to obtain higher percentage of seedlings. Seeds loose viability quickly and their poor germination capacity has augmented the threat for its survival in their natural habitats (Sharma *et al.,* 2000) thus embryo culture can shorten the breeding cycle by overcoming dormancy in seeds. Dormancy may be caused by endogenous inhibitors, light requirements, low temperature, and embryo immaturity (Yeung *et al.,* 1981). Seed dormancy factors may be localized in the seed coat, the endosperm or both. By

removing the embryos from seed to overcome these factors, the embryos germinate and grow quickly and the breeding cycle is shortened (Fathi and Jahani, 2012). In *N foetida*, dormancy is due to physical presence of hard seed coat, physiological conditions like high amount of phenolic compounds (Sharma *et al.,* 2000; Khan, 2013) and abscicic acid (Tejavathi*et al.,* 2011). Thus these factors results in inhibition of germination resulting in poor germination frequency. Phenolics are known to inhibit the enzymes of glycolysis and OPP pathway thereby affecting the respiratory processes of seeds (Muscolo *et al.,* 2001). Hence to overcome the drawbacks related to seed germination in *N. foetida* mature embryos were manually removed and were directly inoculated on the medium supplemented with different growth regulators to obtain plantlets directly. It was noticed that embryo germination was affected by embryo orientation on the medium, where maximum germination rate was recorded when the radical end of embryos were placed upright and not imbedded in the medium and germination was reduced to 50% when embryo meristematic region was fully embedded upright in the medium (Rambabu *et al.,* 2006) and callus formation was recorded at basal tip. Chinese Leymus which has a time-consuming breeding cycle and high level of seed dormancy and to enhance the breeding process, a technique was developed to shorten the breeding cycle by culturing embryos *in vitro* to produce plantlets immediately (Liu *et al.,* 2004). In the present study also experiment conducted to develop plantlet by *in vitro* embryo culturing. Among the different combinations of growth regulators tested embryos inoculated on the medium containing 1.0mg/l Kn + 0.2mg/l NAA resulted in 100% germination.

Conclusion:

From the present investigation it can be concluded that seeds which have hard seed coat and higher amount of phenolics may fail to germinate even at optimum condition. *In vitro* germination and seeds treatment may cause maximum and rapid germination rate. And embryo culture is the only feasibility to overcome seed abortion and poor development of seeds due to germination inhibitors existent in high quantity in the mature seeds.

Fig. 1 *In vitro* seed germination

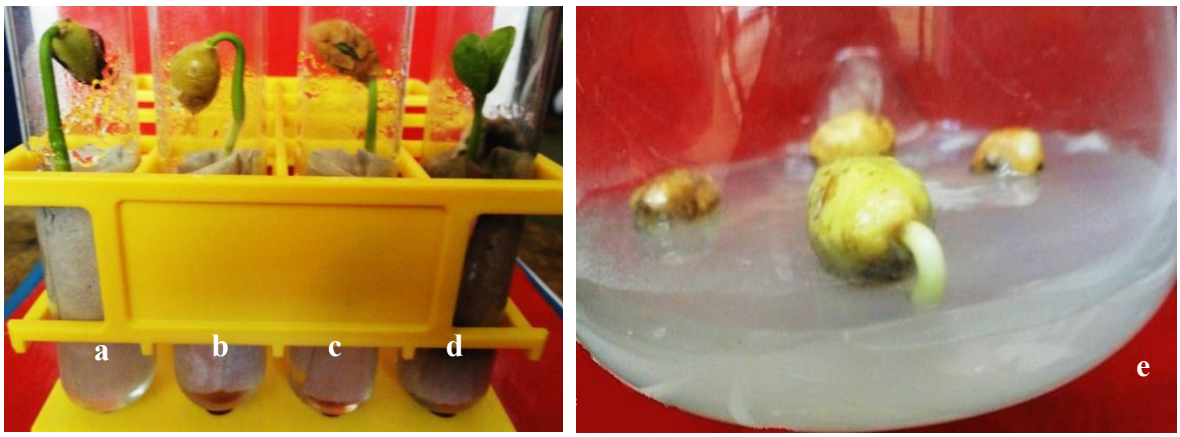


Fig. 1 Seeds inoculated on filter paper bridge for germination **a.** H2SO4 treated normal seeds **b.** H2O2 treated decoated seeds **c.** 20mg/l GA3 treated decoated seeds **d.** 10mg/l GA3 treated decoated seeds and **e.** decoated seeds on half strength MS medium.

Fig. 2 Stages of embryo into complete plantlet

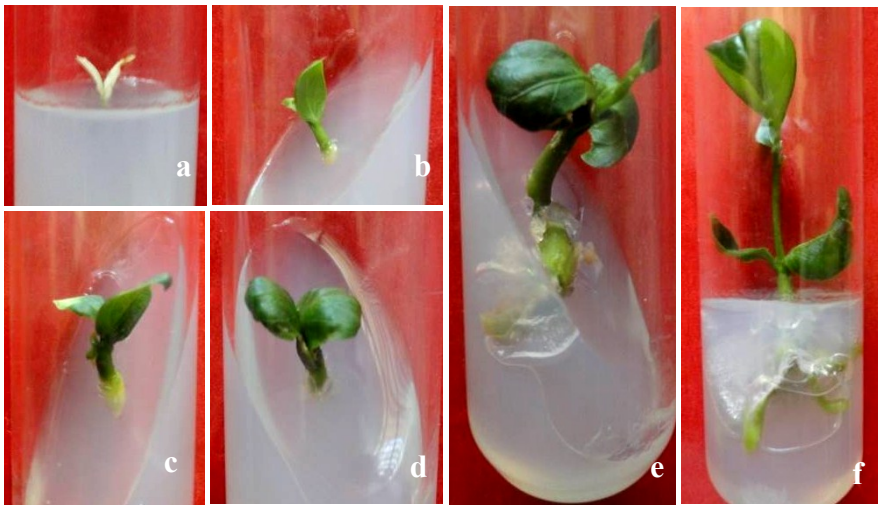
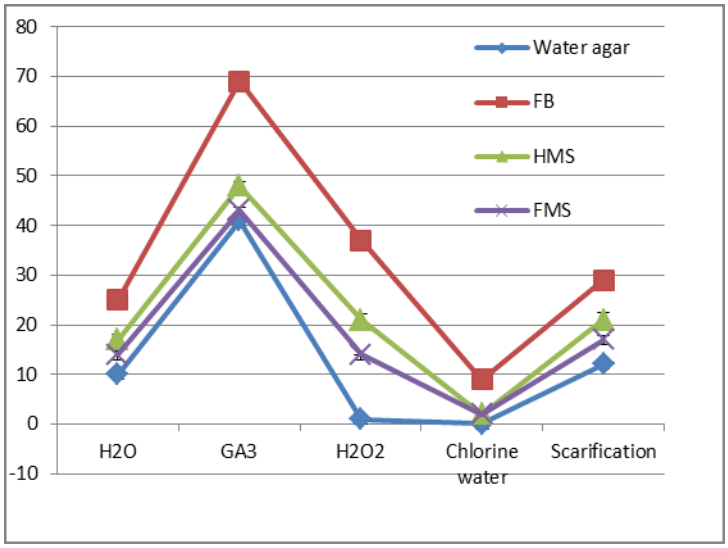
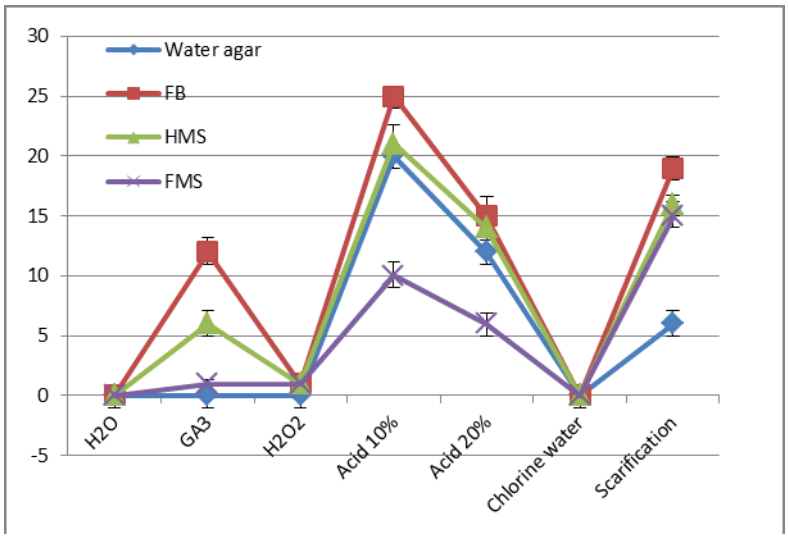


Fig. 2 Differential stages of plantletconversion from embryo dissected out longitudinally splitting the cotyledons and inoculatedon MS medium containing 1.0mg/l Kn + 0.2mg/l NAA **a.** White feathery cotyledons on 3-5th day of inoculationb**.** cotyledons turning to green colour and increase in size on 8-10th day **c** and **d.** Embryo on 15 and 20 day of culture **(Only shoot development till 20th day) e.** Plantlet formation with radical development and **f.** Complete plant with thick roots in 40days of culture.



Graph 1: Effect of different methodologies for *in vitro* seed germination (Decoated seeds)



Graph 2: Effect of different methodologies for *in vitro* seed germination (with seed coat)

Table. 1 Effect of different plant growth regulators on embryo culture of *N. foetida*

Conc. of PGR (mg/l)		**No of days required for initiation of shoots**	**% of plant conversion**
0.0	0.0	19	27±0.67
BAP	**NAA**		
1.0	0.5	10	22±0.58
	1.0	12	24±0.58
	1.5	11	25±0.00
	2.0	11	19±1.15
2.0	1.0	12	36±1.15
	1.5	12	39±2.31
	2.0	13	27±2.60
Kn	**NAA**		
1.0	**0.2**	**7**	**100±0.67**
	0.5	8	94±1.17
	1.0	10	94±0.58
	1.5	8	92±0.58
	2.0	7	92±1.73
2.0	0.2	10	97±0.00
	0.5	10	91±3.18
	1.0	10	89±0.34
	1.5	9	89±0.58
	2.0	7	83±0.89

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