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Conservation of an Endangered Medicinal Plant (*Taxus baccata* L. Subsp. Wallichiana) of Uttarakhand Himalaya through Embryo Culture

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ABSTRACT

The objective of present investigates was to develop seedling emergence and seed dormancy breaking techniques of Taxus baccata, an important medicinal plant which seeds have proved difficult to germinate. Seed germination has been studied from many years to develop a procedure to obtain seedling from those seeds which is inhibited due to dormancy. Propagation through seed is slow and cannot respond to the growing demand of planting material for extraction of Taxol (an anticancer drug). Seedlings of Taxus baccata were obtained by embryo culture to overcome the lengthy phase of dormancy in seeds. The de-pulped seeds were dried under controlled laboratory conditions. Embryos isolated from the 100% sterile seeds are used for embryo culture; absorbent activated charcoal 5gm/L shows better result than without absorbent in embryo germination. The best embryo development was seen in 1000 ppm pretreatment gibbrellin+ Murashige and Skoog media + activated charcoal 5gm/L that is 30%. Murashige and Skoog media + activated charcoal 5 gm/L treatment had the next best treatment for embryo germination that is 20%. Storage times cannot affect the rate of seed dormancy.

Key word: *Conservation, Taxus baccata, Dormancy, Taxol*

INTRODUCTION

Conservation of valuable medicinal plant species is a milestone to restore the population of threatened species to human welfare. *Taxus baccata* is a slow growing evergreen dioecious, non-resinous gymnosperm tree attaining heights up to 10- 15 m, often with multiple trunks and spreading, rounded or pyramidal canopy. Forest conifer species occurring primarily in Garhwal Himalaya ranges 1800 m to 3300m asl in Uttarakhand[1, 21]. It is medicinally important due to taxol, an effective anticancer drug[25]. *Taxus baccata* L. subsp. wallichiana (Zucc.) belongs to family Taxaceae of order Taxales, commonly known as "Thuner" in Garhwal Himalaya.

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Himalayan Yew, a plant that grown in the Himalaya is the only species of Taxus found in India and in Uttarakhand[20] which is found in forest regions with uneven relief and rocky areas and grown in the slopes of mountain with other high altitude plants.

Seed germination is a critical phenomenon in *Taxus baccata* due to a long phase of dormancy (1.5 to 2 years)[24,11]. Indiscrimination of plant for its chemical constituents Taxol and paclitaxel extraction is the main cause of plant deterioration[13, 3, 22]. Taxol (a potent anti-cancer drug) is useful in treatment of ovarian and breast cancer[8 ,26, 18, 6, 12] and also has helpful to cure the patients suffering with melanoma and other solid tumors[27]. The stem, needle, twigs and bark of Taxus species have been reported to contain taxol[28] first isolated from the stem bark of *Taxus brevifolia* a pacific yew[26].

The species is categorized under endangered medicinal plant due to high conservation priority for its medicinal use[9].Germination and regeneration through seed is very complicated and it require a complex treatment like seed in -vitro culture for conservation of Taxus species[29,4,19]. Embryo culture is an alternative tool to overcome from lengthy period of seed dormancy. The objective of present study was, therefore, to establish a method which is helpful to break seed dormancy in *Taxus baccta*, and establish a protocol for dormancy breaking to save the plant for its further use, and to find out the interaction between seed dormancy and seedling development.

MATERIAL AND METHOD

Seed collection: Dark brown matured fruit covered with arils (possessing red colour) of *Taxus baccta* were collected from Joshimath (30.5506° N, 79.5660° E) in district Chamoli Uttarakhand, India. In the month of October 2015, after collection, the mature seeds were rubbed by hand to remove the sticky material (ripened red arils) over the seeds, de- pulped seeds were washed thoroughly with running tap water, undesirable material and light seeds were rinsed off. Seeds were dried in shade at ambient room temperature for two days.

Experimental work: The experimental work conducted in the laboratory, Department of Seed Science & Technology HNB Garhwal University, Srinagar Garhwal, Uttarakhand, India during 2015-2016. Prior to embryo culture test, thousand seed weight, seed length, seed diameter and seed viability was observed. Thousand seed weight were weighed (Anamed digital electronic balance) and average seed diameter and seed length was calculated by using Mitutoyo digital vernier calipers. Seed viability of *Taxus baccata* seeds was determined by using four replicates (50 seeds in each replicate). Seed coat was removed carefully with surgical blade before immersing in1% of 2, 3, 5, triphenyl tetrazolium chloride) solution (5 Grabe 1970,). Dark red stained seeds were considered as viable and vigours.

Embryo culture method: seeds were disinfected by washing with tween -20 under normal tap water and then three times washed off with distilled water and then seeds were immersed for 5 minutes in 70% ethanol for sterilization and then washed three times with sterile distilled water, then the seeds were soaked in 5% sodium hypochlorite (NaClO) for 15 minutes then washed off 3 times with sterile distilled water. Embryos were excised from longitudinally halved endosperm with the help of surgical blade (No 3) and then the sterile embryos were transferred into culture media under aseptic condition in laminar air flow. Excised zygotic embryos from mature seeds were cultured in dark condition for two months in Murashige and Skoog media[10] + activated charcoal (AC) + pretreatment of seeds with 1000ppm gibberellic acid (GA).

Culture media and conditions

Zygotic embryos were cultured on MS basal media. All basal salt media were supplemented with activated charcoal and solidified with 0.7% plant agar. MS media supplemented with activated charcoal is used to observe the effect of basal media on embryo germination, seedling development and used to bind phenolic compounds. Seeds were pretreated with gibbrellic acid to initiate early germination activates of seeds. Embryos were cultured on MS basal medium supplemented

with GA and activated charcoal at different concentration (100 ppm GA and 5gm/l activated charcoal).The pH of media was adjusted to 5.8 before autoclaving at 121°c for 20 min at 15psi. 10 embryos were placed in sterile jam bottles containing 100 ml of culture medium. After transferring embryos in nutrient media, Jam bottle were sealed with parafilm and incubated at 25°c in darkness for about 6 weeks. The germinated embryo cultures were transferred to fresh MS medium (the same formulation), and then maintained under photoperiod of 16 hrs. Every treatment was performed in replicates of ten embryos each.

RESULT AND DISCUSSION

Seed play an important role in germination and rebuilt of regeneration potential, to develop a healthy seedling, and they are the only vehicle to carry genetic information from one generation to another generation. A healthy and viable seed is a key factor to develop a healthy plant population. Morphological characters of seed are the main cause of their survivability and their establishment. Seed of *Taxus baccata* possess a hard seed coat and the average weight of thousand seeds was 62.05 gm, seed length 5.920mm, seed diameter 4.03mm was measured respectively (Table.1).

Table 1: Seed morphological parameters of *Taxus baccta*

Parameters	seed length	seeds diameter	thousand seeds weight
Mean	5.92±	4.03	625.13
sd	0.03	0.04	0.74

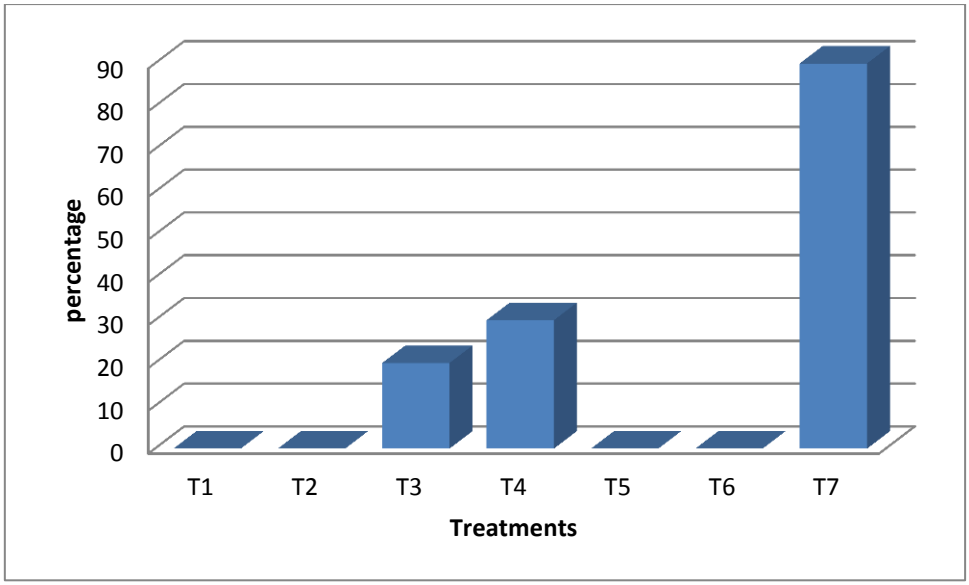

Fig. 2: Seedling development percentage of *Taxus baccta* on MS media supplemented with different media and concentration of growth hormones

Treatments: T1: MS, T2: MS +AC 3gm/L+GA, T3: MS+AC 5gm/L, T4: MS+AC 5gm/l/L+ 1000PPM GA3, T5: MS+ kinetin 5 ppm, T6: ms +AC 5gm/l+ GA100ppm, T7: viability percentage.

Those embryos which cultured in media without activated charcoal did not show any growth. All embryos remained white in colour and there will be no change after transferring the embryos in alternate photoperiod conditions. MS medium containing 5 gm/l activated charcoal showed better embryo growth rather than MS medium without activated charcoal, it showed fewer abnormalities such as seedling browning and twisting. Embryo and seedling showed better growth in media containing activated charcoal because it decreases their browning and bind phenolic compounds. MS media+ Activated

charcoal+ pretreatment of 1000 ppm GA shows good sign of germination in early stage of culture under dark condition. The best embryo development was seen in 1000 ppm pretreatment GA+ MS media+ 5gm/l activated charcoal that is 30% and MS media+ activated charcoal 5 g/L treatment had the next best rank for embryo growth germination that is 20%. Darkness in initial four week culture was reported to improve germination, seedling emergence and development was recorded after two months culturing. Seed germination is epigeal and seeds have two cotyledons with round apices up to 2 cm

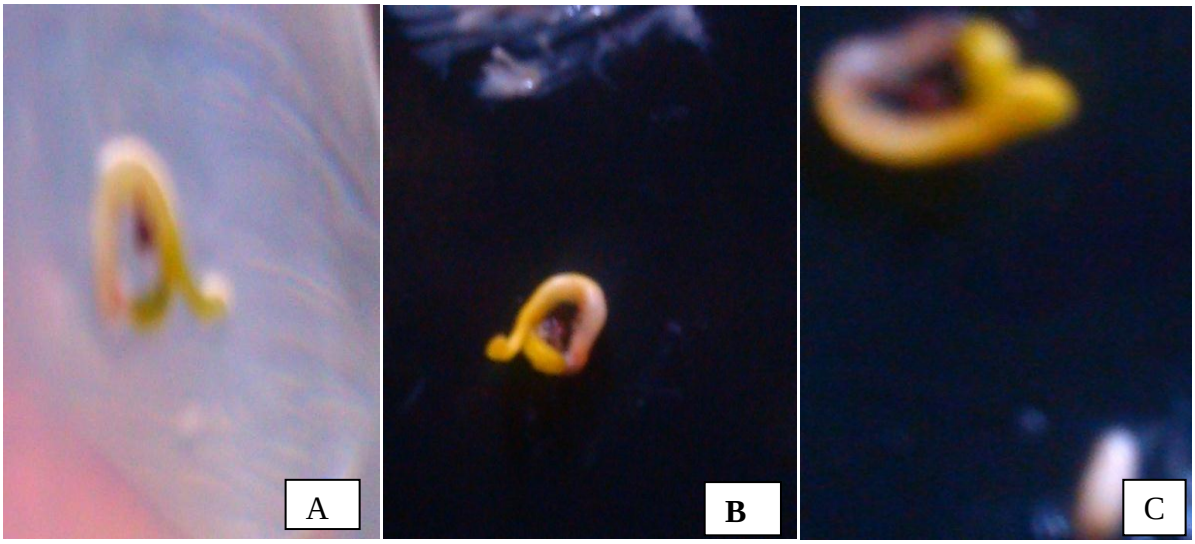

Fig. 2: Germinated embryos of *Taxus baccta* A: germinated seed after 2 month embryo culture. B & C. Seedling emergence in media containing activated charcoal

Seed dormancy is categorized into five classes: physical, physiological, morphological, morphophysiological, and combinationa[2]. Each type of dormancy is influenced by interaction between genetic and environmental factors which are responsible for the inhibition or promotion of embryo growth and seedling development[21]. The seed germination is not directly related with viability because the size of embryo is underdeveloped at the time of seed ripening, and seeds require pretreatment for seed coat imposed dormancy and embryo growth. This type of combinational dormancy is known as morphopysiological[14,15].

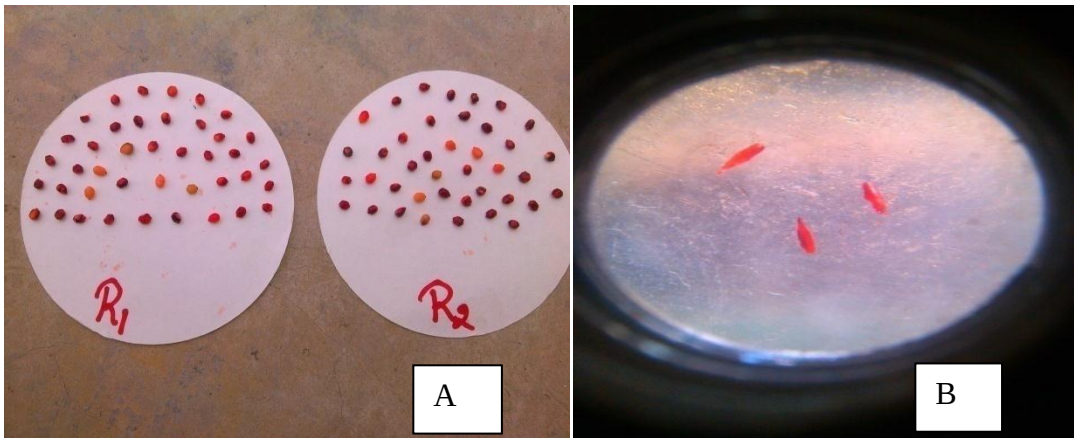

Fig. 1: A viable seeds of *Taxus baccta.* B: microscopic view of viable embryos

Taxus, seed shows 90% viability immediately after collection but in natural seed sowing process seed germination percentage is 0 percent, due to a strong, species-dependent dormancy, presence of an immature embryo in mature seed[16]. In *Taxus chinensis* that few embryos germinated less than 14 hours photoperiod developed into full seedlings whereas more than 20% embryos germinated in continuous darkness grew into full seedling[23].The germination of zygotic embryos of *Taxus baccta* was first described by Le page and Degivoy (1970). The seedling development was 15 to 40 percentages which is related with the result[4, 7] in case of immature of embryos Taxus. A response of mature zygotic embryos was different on different basal cultural media. The basal media formulation has an effect on germination it shows the resemblance of germination percent of *Taxus baccata*.

CONCLUSION

The present observation and result shows that the in- vitro culturing of isolated yew embryo proved to be an efficient procedure for overcoming seed dormancy and inciting seedling growth of *Taxus baccata*. MS medium supplemented with an absorbent to absorb phenolic compounds such as activated charcoal (5 gm/l) yielded the longest root length and shoot height. Embryo culture method plays an important role in regeneration of population in *Taxus baccta* in which natural seeds germination are very poor. Regarding above mention results MS+ AC 5gm/l + GA 1000 ppm pretreatment appeared to be most effective treatment for successful embryo culture. In conclusion the ability of developed seedling and their quality depends on the maturation phase of seed, enlargement of embryo and basal media used for embryo germination. Therefore preserving genetic diversity of *Taxus baccata*, the embryo culture technique provides a quick and reliable method for colonel propagation and plant conservation.

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