Native Indigenous Tree Species Show Recalcitrance to in Vitro Culture

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Abstract

The status of the Mauritian forest is alarming with deforestation and invasive alien species deeply affecting the indigenous flora. Therefore, major conservation strategies are needed to save the remaining endemic tree species. Explants form three endemic tree species Elaeocarpus bojeri, Foetidia mauritiana and Sideroxylon grandiflorum were grown under in vitro conditions. These species are rareand Elaeocarpus bojeri has been classified as critically endangered. Thidiazuron (TDZ) and 6-Benzylaminopurine (BAP) were used as growth promoters in order to stimulate seed germination, callus induction. Half strength Murashige and Skoog's (MS) media supplemented with coconut water, activated charocoal and phytagel were used as growth media. Hormone levels of TDZ were 0.3mg/l and 0.6mg/l while BAP level was at 1mg/l. Germination rate for E.bojeri was low (5%) with TDZ 0.3mg/l. Sideroxylon grandiflorum seeds showed no response to in vitro culture, while F mauritiana showed successful callus induction with TDZ 0.6mg/l and 0.3mg/l.

Keywords: *Elaeocarpus bojeri, Foetidia mauritiana, Sideroxylon grandiflorum, in vitro* culture, TDZ, BAP

1.0 Introduction

The increase in population size, island development and sugarcane cultivation led to drastic deforestation that reduced the native forest to less than 2%. Mauritius has the most endangered terrestrial flora in the world according to the IUCN (Ministry of Environment & Sustainable Development (MoESD), 2011). Human activity pressure for 370 years has deeply affected the ecosystem of Mauritius (Smith and Fisher, 2009; Florens et al., 2012). The latest figures from the Fourth National Report on the Convention on Biological Diversity in August 2010 stated that Mauritius houses 685 species of indigenous flowering plants of which 267 are endemic. Mauritius has 6 endemic plant genera and 150 endemic to the Mascarene Archipelago Alarmingly, 89% of the endemic plants are classified as threatened and 61 of the indigenous species are extinct.

Elaeocarpus Bojeri is a critically endangered plant listed in the 1997 lUCN Red List of Threatened Plants (Walter and Gillett, 1998; Page, 1998)]. Also known as "Bois dentelle" the native *E. bojeri* found in Mauritius gives beautiful white flowers and fleshy curved fruits. *Sideroxylon grandiflorum* also commonly called " Tambalacoque was classified as a rare species in Mauritius. Four species of the genus Sideroxylon : *S. cinereum, S. grandiflorum, S. puberulum, S. sessiliflorum* (Florens et al., 2012) are found in Mauritius. *Foetidia mauritiana* is a tree species listed in the conservation priority of the Forestry Service (Mauritius). *Aristotelia chilensis* (Elaeocarpaceae) was propagated by seeds giving a success rate between 34 to 63 % while through vegetative propagation using stalk cutting gave 100% success rate with IBA hormone (Ogunsola and Ilori, 2008).Successful embryo culture of *Synsepalum dulcificum* (sapotaceae) were obtained when treated with low level NAA, BAP and IBA hormones (Dalila et al., 2013). *Barringtonia racemosa* (Lecythidaceae) was micropropagated through callus to plantlets using endosperm and leaf explants (Saad and Elshahed, 2012). The hormones used were 2,4-D and Kinetin at different concentrations. Coconut water supplemented in culture media act as a promoting factor and enhance the *in vitro* performance (Wang and Huang, 1976; Aina et al., 2012) of developing embryo. Activated charcoal added in culture media aids to overcome growth inhibition by chemical released due to stress and do also mimic soil characteristics (Shadang et al., 2006).

TDZ is thought as a mysterious hormone with its ability to have both auxin and cytokinin. Although its mode of action is still unknown, it was observed that TDZ induces and enhances biological activities in a plant cell. It is believed that TDZ is the best cytokinin as it has the ability to cause propagation of recalcitrant species Compared to other phytohormones, TDZ is effective at concentration 10 to 1000 times lower. Incredibly, TDZ has a wide range of effects over an impressive diversity of plant families such as shoot formation, somatic embryogenesis, seed germination, callogenesis and even flower induction depending on the explant used (Trivedi et al., 2010). Germination is a cascade of events which start with the uptake of water and terminate with the radical extension (Bewley, 1997; Kranner et al., 2010). The process involves a high metabolic rate with mobilization of food reserves. However, even dormant seeds can go through all the metabolic steps and yet the radical fails to elongate. In order to avoid the problem of seed dormancy and the inhibitory effects which prevent the embryo from developing, embryo rescue can be very efficient. This technique promotes the growth of an immature or weak embryo and has been commonly used to produce hybridized plants in which embryo abortion is a problem (Reed, 2004). The embryo benefits through direct contact with the media and away from the inhibitory effects from its surrounding tissues.

1.2 Material and Methods

The fruits of *E. bojeri* and *F. mauritiana* and *S. grandiflorum* were kindly provided by the Forestry Service (Mauritius).Modified Half strength MS was prepared according to Saad and Elshahed (2012). The standard constituents of the media used were ½ strength MS, 0.8g of phytagel and 10% coconut water. The coconut water was heated to 80ºC to precipitate proteins and filtered with Whatman filter paper. Two batches of media were prepared which consisted of activated charcoal (3g/l) (Wand and Huang, 1976) and activated charcoal free. Sterilizing protocol were adapted from Kishore *et al.*, (2010) and Zhihui *et al.*, (2009).The cultures were maintained at a temperature ranging from 22-24 °C. Moreover, the light exposure is set to be 16 hours of light and 8 hours of darkness. The light intensity under which the explants grew ranged from 1.12klux to 1.79klux of light. Germination rates of different explants were assessed using Digital photography. Scales were stuck on fixed position in each culture pot, and digital photographs were taken at the same position atregular intervals. It should be noted that all culture pots were purchased from the same supplier to ensure minimal effects of refractive index of glass. These photographs were processed in the software Scion Image and mean length and areas were calculated as described by Bhoyroo .V *et al* (2011). SPSS 16.0 was used for statistical tests.

1.3 Results

E. bojeri seeds were inoculated in two batches. Throughout the experiment, the testa colour, seed enlargement and testa splits were observed for both batches. The best results obtained were the emergence of the radical and plumule with TDZ 0.3mg/l and TDZ 0.6mg/l after 2 months of inoculation. The seed area was more informative for the growth of seeds. Differences among different hormonal treatments can be observed in Figure 1. The control and the BAP batch both started with a decrease in area which continued for the BAP batch but not for the control. BAP treated seeds had a total loss in area of 10.85mm^2. The control seeds gained 14.002mm^2 in area and the highest mean area measured is 48.678mm^2(S.E: ± 13.83071). TDZ had a sharp continual increase over 2 months gaining 22.916 mm^2 with a highest mean of 59.236 (S.E: ± 19.30031. No significant difference in growth between the three treatments were observed (ANOVA, P>0.05)

After inoculation of embryo of *F. mauritiana*, callus were obtained and these were sub-cultured. First month after innoculation of callus showed a decrease in the size (Figure 2). The control batch had a steeper decreasing trend of 1.92mm^2 while TDZ 0.3mg/l decreased by 0.69mm^2. However, TDZ 0.6 mg/l showed a small increase in size of 0.2mm^2. The second month showed an overall increase in growth except for the control. TDZ 0.3mg/l and TDZ 0.6mg/l batch showed a significant increase in growth in the third months. An overall decrease in the size of the calluses in all treatments was measured after the 4th month. This observation shows that, the *Foetidia mauritiana* callus grow best for 2 months and then need to be sub-cultured for continuity of growth. No significant difference were observed among the different treatments (Kruskal-Wallis Test, p>0.05). Embryo of *Sideroxylon grandiflorum* showed an overall decrease in size over the growth period for all treatments.The area also increased to its maximum in week 2 by gaining a total of 5.702mm^2 and a maximum area of 58.108mm^2 (S.E: ± 7.422807). Although, TDZ 0.3mg/l was only hormone concentration to give positive increase in measurement in the second month, it then decreased. No significant difference were observed between the treatments (Kruskal-Wallis, P>0.05) for each measurement.

1.4 Discussion

Fewer than 4 seeds per fruits were obtained for *F. Mauritiana* and most seeds were non viable. Callus propagation from mother callus explants were obtained with TDZ 0.6mg/l and TDZ 0.3mg/l. Moreover, these results correlate with several other studies performed using TDZ such as Vila et al., (2003) who showed that the best regeneration of *Melia azedarach* somatic embryos were obtained with TDZ concentration of 4.54µM (1mg/l) after 6 weeks. In a study performed by Trivedi et al (2010), *Aspargus recemosus* grew best on TDZ 0.4mg/l and took 28 day for callusing (40.33%) while on concentration of 0.2 and 0.8mg/l, nearly 2 months were needed to obtain 28.25% and 25.15% callusing. The drastic decrease in area in the third month could be due to the effect of TDZ which kill or hinder the growth of callus after 2 months, as claimed by Trivedi et al (2010). In comparison to the other studies, *F.mauritiana* grew more rapidly at a higher dose of TDZ. However, the callus should be treated for a maximum of 2 months and then sub-cultured on hormone free media.

S. grandiflorum seeds in this study failed to germinate when treated with hormone free and TDZ supplemented media. Even though fissures were observed, no germination occurred. Although conversations with Forestry service officers stated that the orientation of the seed in the soil is a determining factor in germination, no information about the position of the cotyledon *in vitro* was obtained. Thus, when the testa was broken, the same position of the cotyledon in the seed coat was applied *in vitro*; the ventral part of the cotyledon faced the media while the upper part was exposed to light and oxygen. Several studies support the importance of seed orientation such as Jaskani et al (2006) on germination of watermelon seeds showed that 100% cotyledons emerged without seed coat with radical up orientation compared to horizontal seed orientation. A second study conducted by Mishra et al (2013) on the *in vitro* germination of *Pterocarpus marsupium* Roxb. showed the importance of media composition and seed positioning. In the horizontally positioned batch, higher germination percentage was obtained for half MS (78.23%) compared to full MS (70.39%). More importantly, Bosy and Aarssen (1995) proved that there was a significant difference in seed positioning with the seed they used. Other reasons stated that could account for the inability of the *S.grandiflorum* seeds to germinate are; the localized light receptors on the seed being blocked, oxygen diffusion occurring at specific regions in the seed coat. An alternative tool used to initiate germination was embryo rescue. Activated charcoal was used to absorb noxious substances that could cause embryo dormancy as white secretions on the seeds and gelatinous substance surrounding the embryo were observed. Several factors stated by Mehetre and Aher (2004) could be responsible for the inability of the embryo to germinate such as age (developmental stage of embryo), temperature and media composition. Other considerations stated by Reed (2004) such as time of culture, light and pretreatments should be taken into consideration for further experiments involving *S.grandiflorum* embryos.

Elaeocarpus bojeri seeds gave the best results in TDZ at 0.3mg/l and 0.6mg/l concentration after 2 months of inoculation. After inoculation, a black secretion was observed in the phytagel. It was believed that it was a phytochemical being release due to environmental stress. Thus activated charcoal was used in the 2nd batch as preventive measure if ever the phytochemical was inhibitive. The growth pattern of the seeds from the 1st and 2nd batch followed a 3 phase growth stated by Bewley (1997). The morphological observations showed that most seeds completed phase 1 and phase 2 that is rapid water uptake which correlated with an increase in area and width, solutes leak such as the black substance observed in the gel and an increase in respiration observed by the formation of water droplets inside the pots. Although the phase 2 involved synthesis of proteins and mRNAs which cannot be observed, an indication of growth was seen by the slow increase in size and the opening of the small fissures. However, most seeds stop or remained in phase 2 without entering in phase 3 which is called post germination . Prior to radical extension, a drastic increase in size of the seed was observed which caused the testa to stretch and break allowing enough space for the cotyledon and radical to emerge. This correlates with 1) a drastic uptake of water and (2) radical extension is a turgor driven process. Furthermore, the third hypothesis stated by Bewley (1997) is also in line with the germination of *E.bojeri* seed that is the weakening of the surrounding tissues of the radical was necessary for the elongation of the radical tip. This could be a potential reason for the low germination percentage of *E.bojeri* seeds. In addition, several seeds did not grow in size the same way as the other seeds. This is due to several reasons such immature seeds, seed dormancy and even accumulation of abscisic acid (ABA). ABA normally accumulates during mid development and synthesis of storage reserves. Lastly, an interesting observation was the emergence of albino cotyledons. Albinism is due to the loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes (Kumari et al., 2009).

Several factors that could account for the 2 albino results obtained are; (1) Environmental factor such as light intensity, temperature, media composition (sugar percentage) and (2) Genetics. Despite the environmental factors, the genetics of albinism is much more important. Albinism is a recessive trait controlled by many loci. As a result of disparities between nuclear and chloroplast genomes, albinism occurs due to defective pigments formed. Faster growth rate and firm results were obtained with TDZ hormone such as the first in vitro seed germination of *Elaeocarpus bojeri* and callus growth of *Foetidia mauritiana*. *Sideroxylon grandiflorum* failed to germinate *in vitro* even with the use of embryo rescue technique. This suggests that some of the tree species do show some recalcitrance to *in vitro* response.

1.5 Acknowledgements

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1.6 Cited Literature

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1.7 List of Figures

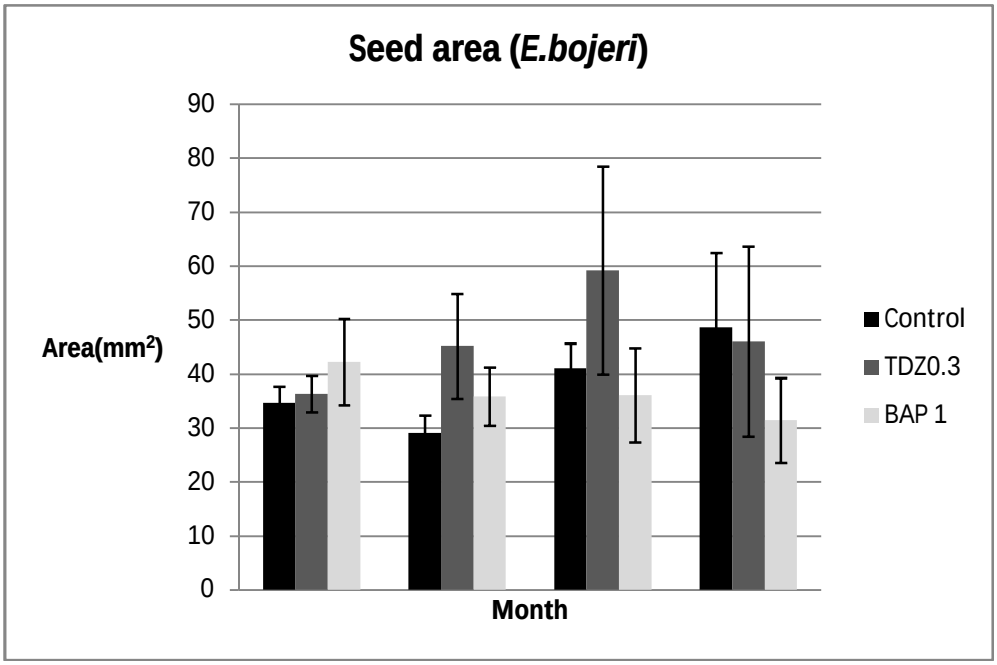

Figure 1: Growth Rate (mm^2) of E. bojeri in three Hormonal Treatments

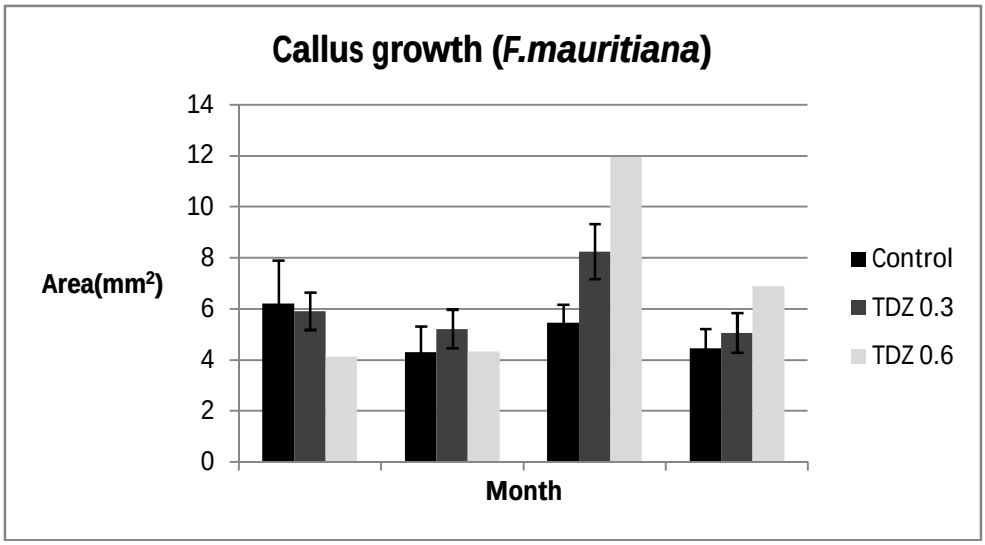

Figure 2: Growth Rate (mm^2) of F. Mauritiana in three Hormonal Treatments

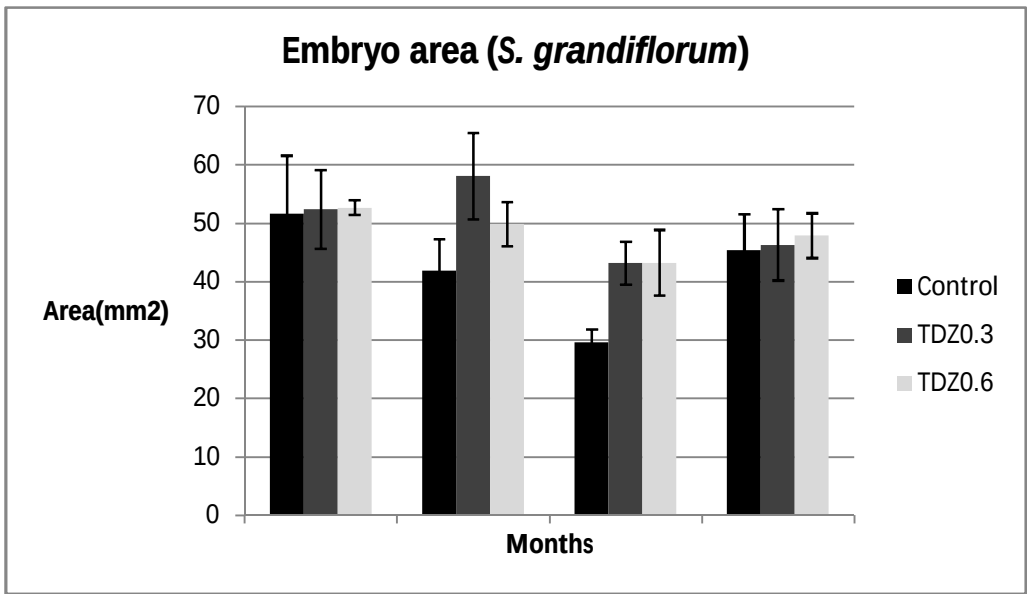

Figure 3: Growth Rate (mm^2) of S. grandiflorum in three Hormonal Treatments