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In vitro seed germination and development of *Butea monosperma* (Lam.) Taub. Var. *lutea* (Willt.) : a step for rehabilitation

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Abstract

Enhanced in vitro seed germination, seedling development protocol has been established for conservation of very rare and globally endangered woody tree species, Butea monosperma (Lam.) Taub. var. lutea (Witt.) Fabaceae). It differs from Butea monosperma var.monosperma in presence of ivory- white/ bright yellow flower buds and flowers. The plant seeds very low viability and ex vitro germination rates responsible for its rare and globally endangered status. Mature seeds were cultured on two basic inorganic media with full (F) or ½ strength (H) of MS or WPM supplemented with various concentrations of N6-benzyladenine (BA, 2.22, 4.40, 6.62 and 8.40 µM) or thidiazuron (TDZ, 0.45, 2.27, 4.54, 6.80 µM) alone. Within the range evaluated, highest percentage (65%) of the seed germination (pre-hot water treated for 3 min) was obtained from seeds cultured on MSF medium containing 4.40 µM BA . The same medium has promoted the highest growth of seedling with a mean of 3.57 ± 0.06 cm shoot length and 2.44 ± 0.17 cm root length after 3 weeks of culture. BA at 6.62 µM level in WPMF medium provoked 50% seed germination response. While, TDZ (2.27µM, 0.45 µM) induced 45% and 30% response on WPMF or WPMH medium respectively. The seedlings (90%) of B. monosperma var. lutea readily acclimated to greenhouse conditions. The present investigation for the first time describes an enhanced in vitro seed germination and seedling development protocol for conservation and restoration of B. monosperma var. Lutea.

Keywords : *BA; endangered ; in viable seeds ; pre-hot water*

1. Introduction

Butea monosperma (Lam.) Taub. var. *lutea* (Witt.) Fabaceae) possess charismatic ivory-white (albino) flower buds and yellow flowers (Fig. 1A). It is taller than well know *Butea monosperma* var.*monosperma* (Flame of the forest), which has elegant orange-scarlet raceme inflorescence (Fig. 1B). The plant is much demand in folk medicine. Stem bark extract with jeera powder used for leucorrhoea, jaundice and skin diseases. The decoction of stem bark is said to be given as a tonic to women after child-birth. One teaspoonful of root bark juice can be given orally a day for three days as contraceptive [1]. Chemical screening of the parts of the species has shown the presence of flavonoids, chalcones, linoleic acid and unsaturated fatty acids [2].

It is also different from well-know *Butea monosperma* having cyclitols which are important compounds know to heal asthma and chronic bronchitis. Flowering and fruiting were observed once in two years. *B. monosperma* var. *lutea* is endemic to Deccan plateau of India. It has very less population i.e., equalled or less than 100 plants across the plateau. The species has been reported from around Aurangabad in Maharashtra, Jillella block of Sirisilla forests of Karimnagar, Peddagutta of Nizamabad and Kummarigudem and Mallakpally of Warangal district, Andhra Pradesh [3, 1]**.** It has been overlooked taxon and finds no mention in the Flora of Andhra Pradesh [4]. It has not been incorporated into the RED DATA Books of India [5] and not yet included in IUCN RED list also. It is very rare and declared as globally endangered medicinal plant by Conservation Assessement Mangement Planning Workshop for Medicinal Plant of Andhra Pradesh [6].

Currently, the albino variety in the wild facing threat of extinction due to destructive harvesting of plant parts for medicinal use, for fire wood, devastation of its natural habitat and due to lack of knowledge about its rarity. Further, propagation of this plant through seed is hampered by lower rates of the germination as well as viability. Almost all seeds sown in experimental garden were found in viable to reflect on its rarity in nature [7]. For conservation purposes, seed is a source of plant

Table 1 Effect of various concentrations of BAP Or TDZ on *in vitro* seed germination (pre-hotwater treated for 3min) and seedling developement of *B. monosperma var. Lutea*

	A MSF			B MSH			C WPMF			D WPMH		
Hormone conc. (µM)	Seed germination %	Shoot length	Root length	Seed germination %	Shoot length	Root length	Seed germination %	Shoot length	Root length	Seed germination %	Shoot length	Root length
0	NR	0.00 ±0.00a	0.00 ± 0.00 a	NR	0.00 ± 0.00 a	0.00 ± 0.00 a	NR	0.00 ±0.00 a	0.00 ±0.00 a	NR	0.00 ± 0.00 a	0.00 ± 0.00 a
BAP 2.22	35	2.86 ± 0.21 d	1.75 ± 0.29 c	20	2.22 ± 0.07 c	1.80 ± 0.50 b	30	2.06 ±0.50 c	0.95 ± 0.48 b	15	1.86 ± 0.56 c	1.00 ± 1.01 c
BAP 4.40	65	3.57 ± 0.06 d	2.44 ± 0.17 d	40	3.07 ± 0.23 c	2.15 ± 0.42 c	45	2.80 ±0.38 c	1.25 ± 0.68 c	20	1.97 ± 1.19 c	1.25 ± 0.64 c
BAP 6.62	45	1.86 ± 0.14 c	1.55 ± 0.08 c	25	1.96 ± 0.19 b	1.85 ± 0.18 b	50	1.06 ±0.32 b	1.50 ± 0.49 c	30	0.76 ± 1.23 b	0.65 ± 0.11 b
BAP 8.90	20	1.00 ± 0.51 b	0.86 ± 0.13 b	15	1.15 ± 0.32 b	1.66 ± 0.44 b	20	1.07 ±0.11 b	0.36 ± 0.59 b	20	0.60 ± 0.99 b	0.46 ± 0.91 b
TDZ 0.45	25	1.55 ± 0.24 c	0.75 ± 0.43 b	20	2.35 ± 0.43 c	0.95 ± 0.24 a	25	1.25 ±0.49 b	1.22 ± 0.13 c	30	1.25 ± 0.78 c	0.75 ± 0.32 b
TDZ 2.27	30	2.70 ± 0.11 d	1.34 ± 0.08 c	30	2.15 ± 0.08 c	1.94 ± 0.18 b	45	1.98 ±0.61 c	1.15 ± 0.66 c	25	2.40 ± 0.15 d	1.88 ± 0.08 c
TDZ 4.54	45	1.40 ± 0.04 c	1.00 ± 0.11 b	40	1.70 ± 0.16 b	2.00 ± 0.11 c	40	1.50 ±0.19 b	1.00 ± 1.05 b	20	1.20 ± 0.45 c	1.10 ±1.22 c
TDZ 6.80	20	0.97 ± 0.06 b	0.58 ± 0.87 b	10	1.97 ± 0.09 b	1.28 ± 0.43 b	15	1.00 ±0.33 b	0.36 ± 0.59 b	5	0.77 ± 0.31 b	0.70 ± 0.47 b

Values of Mean ± SE of 40 seed explants. In each column means followed by the same superscripted letter did not differ significantly at P≤0.05 according to Duncans multiple range test.

NR- no response

A) MSF - MS medium full strength
B) MSH - 1/2 strength of MS medium
C) WPMF - WPM medium full strength
D) WPMH - 1/2 strength of WPM

material as compared to vegetative material is preferable because it has a wider genetic base. When conventional methods produce low or no germination, *in vitro* techniques can greatly enhance germination [8]. The media composition, qualitative and quantitative aspects of plant growth regulators play a vital role in plant *in vitro* studies. Therefore optimization of these conditions is a prerequisite for *in vitro* plant tissue culture studies.

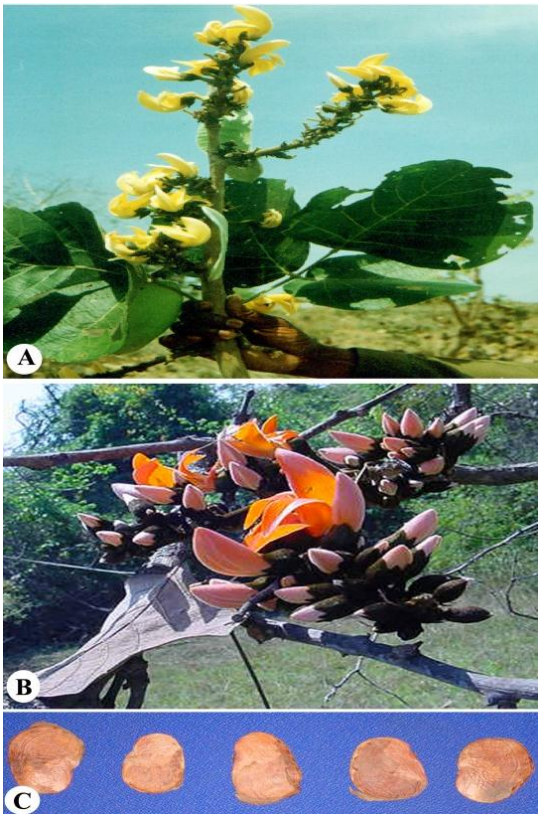

Figure 1 - Exvitro grown Varieties of *Butea mosperma*

A) *Butea monosperma* (Lam.) Taub. var. *lutea* (Witt.)
B) *Butea monosperma* var.*monosperma*
C) Mature seeds of *Butea monosperma* (Lam.) Taub. var. *lutea* (Witt.)

Development of protocols for *in vitro* seed germination and seedling development can be used to rapidly produce large number of *Butea monosperma* var. *Lutea* for germplasm conservation and restoration. There are no reports are available describing seed germination protocols for *Butea monosperma* var. *lutea* that made us interested to develop a simple, reproducible and enhanced *in vitro* seed germination and seedling development protocol for globally endangered and valuable woody plant species. We describe first time a simple, reproducible and enhanced *in vitro* seed germination and seedling development protocol of *Butea monosperma* var. *lutea* using mature seeds.

Materials and Methods

The mature seeds of *B. monosperma* var. *lutea* were collected from a 10 yr old single tree **(**Fig. 1 C) during May to July from plants grow in Jillella block of Sirisilla forests of Karimnagar, Andhra Pradesh, India. Collected seeds were initially washed under running water tap for 5 min, following immersed in hot water (100 ^{0}C) for different time periods (1, 2, 3 and 4 min) to break dormancy (here after called as "pre-hot water treatment") and were soaked in sterilised distilled water (SDW) at room temperature for overnight. The soaked seeds were surface sterilised with 0.1% (w/v) $HgCl_2$ (0.1% w/v) for 4-6 min followed by 4-5 rinses (each of 10-15 min) with sterile distilled water (SDW). After rinsing for 4-5 times with SDW, the seeds were aseptically blotted on Whatman paper and transferred to screw capped bottles (10x8.5 cm) containing 50 ml of two different basic inorganic medium in full and half strength of Murashige and Skoog [9] full strength medium (MSF), half (½) strength of MS (MSH) medium, Woody Plant Medium [10] full strength (WPMF) and half (½) strength of WPM WPMH) containing 3 % sucrose(w/v) and 0.8% (w/v) agar (Himedia, India). All the foresaid medium were supplemented with various concentrations of Plant Growth Regulators (PGR's) N6-benzyladenine (BA, 2.22, 4.40, 6.62 and 8.90 µM) or thidiazuron (TDZ, 0.45, 2.27, 4.54 and 6.80µM) alone for *in vitro* seed germination and seedling development (Table 1 – A, B, C, D).

The seed explants were cultured for 3 weeks on the foresaid media using 25 ± 2 ^{0} C with 16h photoperiod, under white fluorescent light (65 µE/m^2/s). All the foresaid medium without cytokinins (BA or TDZ) and untreated (not pre-hot water treated) mature seeds served as control. After germination, individual seedlings after 3 weeks of culture (plantlets) were removed from culture jars and washed thoroughly with water potted in plastic jars containing sterilized soil and vermiculite (1:1)

mixture. Plantlets were covered with polyethylene sheets to minimize loss of moisture and transferred to green house (28^0 C day, 24^0 C night, 65% RH).

All media pH were adjusted to 5.7 before adding agar (0.8%, w/v, Himedia) and autoclaved at 121^0 C for 15 min. Percent response and mean average lengths was evaluated on the basis of the final seed germination and time taken for germination to commence to seedling optimal growth in 3 weeks of culture**.**). For *in vitro* seed germination/ elongation and rooting, 10 seed explants were used in each of the two replicates for each treatment and the experiment was repeated twice. All the data were subjected to analysis of variance followed by Duncan's multiple range test a (DMRT) for mean comparison ($P < 0.05$).

Results

During our investigation, culture conditions were established for *in vitro* seed germination and development from mature seeds of *B. monosperma* var. *lutea.* All untreated or pre-hot water treated mature seeds at different time periods (1, 2, 3 and 4 min), up on culture on two basic inorganic medium in full or half (½) strength of MS and WPM supplemented with various levels of cytokinin BAP or TDZ alone, showed varied *in vitro* seed germination and development response(Table 1).

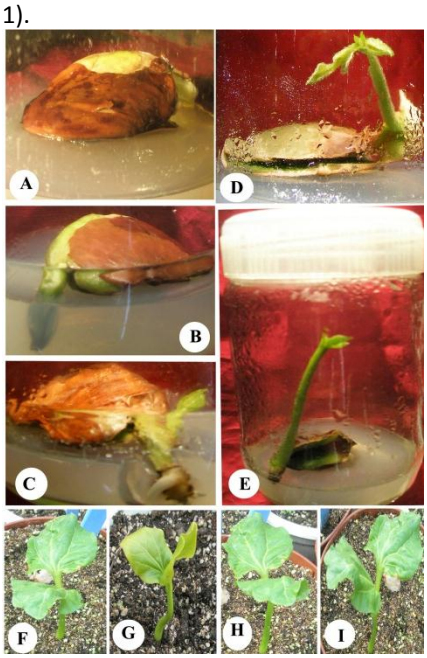

Figure 2 – *In vitro* seed germination and seedling development of *Butea monosperma* (Lam.) Taub. var. *lutea* (Witt.)

A) Cultured seed explants (after 3 d) on MSF medium supplemented with 4.40µM BA

B) , C) , D) Different stages of seedling development on on MSF medium supplemented with 4.40µM BA

E) Developed seedling after 3 weeks of incubation on MSF medium supplemented with 4.40µM BA

F) , G), H) and I) Plantlets on soil in green house.

All the seeds germinated, resumed to seedling which significantly differed in the shoot and root length. No response (germination and development) was observed form the seed explants after 3 weeks of culture. Similar PGRs levels (BA/TDZ) were used in all the set of experiment trails**.** No response was observed from the seeds cultured on media devoid of PGRs (control).

In the first set of experiment (Table 1 A, MSF) seeds cultured on media supplemented with either with BA or TDZ induced 20-65% *in vitro* seed germination. The medium supplemented with 4.40µM of BAP evoked optimal (65%) seed germination response, was also effective in terms of shoot length (3.57 ± 0.06) as well as root length (2.44 ± 0.17) of seedlings within 3 weeks of culture. The seeds cultured on this medium responded after 2 days of culture (Fig. 2 A). Initially, primodial roots of seeds penetrated into medium after 4 days of culture (Fig. 2 B).

Such seeds developed shoots with primordial leaf were elongated (Fig. 2 C, D), adequately to form complete seedling ready for hardening (Fig. 2 E). Reduced response (20%) was observed on medium supplemented with higher levels of BAP (8.90 µM) or TDZ (6.80 µM). Under these conditions seeds developed with mean least average root (0.97 ± 0.06) and (0.58 ± 0.87) shoot length at level of 6.80 µM TDZ. Followed by it, similar seed germination percentage (45%) was observed at levels of BAP (6.62 µM) and TDZ (4.54 µM). BAP at level 2.22 µM produced only 35% seed germination, but the shoot length (2.86 ± 0.21) was second highest when compared to response given by the optimal level of BA (4.40 µM).

In other set of experiments (Table 1 B, MSH), the *in vitro* seed germination response was ranged from 10 to 40 %. The response remained less significant as compared on MS full strength (above set of experiments). However, highest seed germination (40%) was obtained at level of 4.40 µM BAP or 4.54 µM TDZ. Followed by it, another similar response (20%) noticed on media having a level of 2.22 µM BA or 0.45 µM TDZ. BA at 8.90 µM level supported 15% seed germination response with significant reduction in shoot length. Lowest seed germination (10%) was obtained at 6.80 µM level TDZ. Even though, over all seed germination under these conditions comparatively less to the above set of experiments, but the average mean shoot or root length of seedlings cultured on this media (½ of MS) were highest .

In another set of experiments (Table 1 C, WPMF**),** the percent *in vitro* seed germination response under these conditions was ranged from 15 - 50%. The cytokinin BA (6.62 µM BAP) induced 50% response with 1.06 ±0.32 and 1.50 ± 0.49 shoot and root length. Followed by it, similar percent (45%) response was noticed on medium containing 4.40 µM BAP or 2.27 µM TDZ. Under these levels highest shoot and root length was obtained on medium with 4.40 µM BAP. Lowest seed germination (15%) with shortest seedlings having 1.00 ±0.33 and 0.36

± 0.59 shoot and root length was obtained at 6.80 µM TDZ.

In the last set of experiments, seeds cultured on WPM ½ strength (Table 1 D, WPMH**),** with various BAP or TDZ levels showed 5-30% of *in vitro* seed germination response. Among all the set of experiments, this set of experiment have showed reduced response. At 6.80 µM level TDZ lowest percent (5) seed germination was noticed. BAP at 6.62 µM or TDZ at 0.45 µM levels exhibited similar highest seed germination (30%) response. Seeds germinated on medium supplemented with 6.62 µM BAP, 8.90 µM BAP or 6.80 µM TDZ levels given seedlings with shortest shoot and root lengths and roots. However, seeds cultured on medium with 2.27 µM TDZ induced seedlings with highest shoot (2.40 ± 0.15) and root lengths (1.88 ± 0.08).

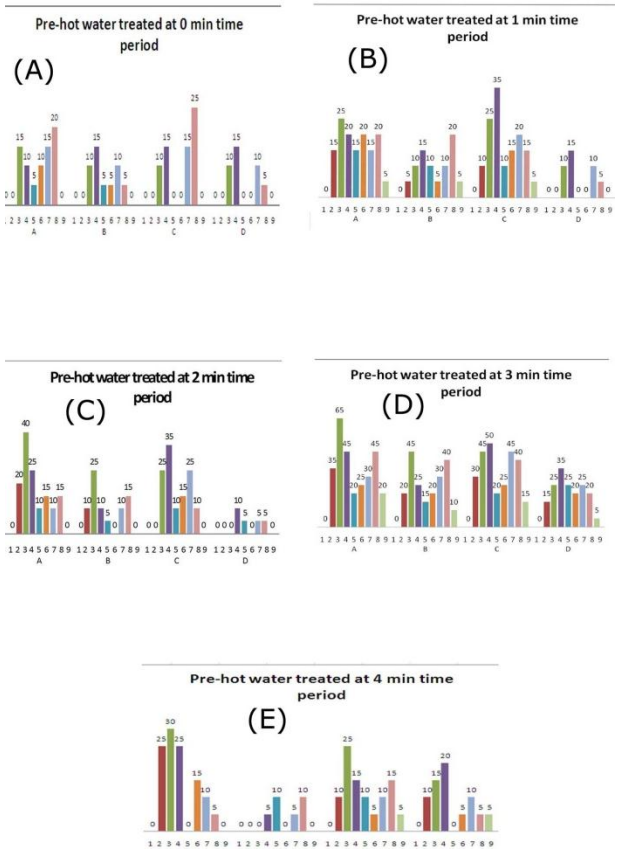

Figure 3 – Effect of pre-hot water treatment at different time periods on percent *in vitro* seed germination.

A) 0 time ; B) 1 min ; C) 2 min ; D) 3 min ; E) 4 min

Seeds exposed to pre-hot water at time periods of 1, 2, 3 and 4 min germinated at a range from 0-65%, while untreated seeds cultured on PGR's supplemented media germinated from 0-25% under similar culture conditions. Pre-hot water treated seeds for a time period of 3 min has produced optimal response in terms of *in vitro* seed germination (20 -65%) and seedling development after 3 weeks of culture (Table 1 A, B,C & D/Fig. 3 D). Whereas, the effect of (100 ^{0}C) pre-hot water tested at 0, 1, 2 and 4 min time periods study has showed reduced *in vitro* seed germination and seedling development response (Fig. 3 A, B, C & E). Increasing severity of pre-hot water treatment (time period 1, 2, 3 min) improved germination rate tired up to a point where seed mortality became apparent (4-5 min). All the seedling developed after 3 weeks were hardened in soil followed by transfer to the green house for further growth (Fig. 2- F, G, H & I). The survival of the plants under green house conditions was 90%.

Discussion

The purpose of this study was to develop on *in vitro* seed germination and seedling development protocol from mature seeds of *B. monosperma* var. *lutea*, a critically rare and globally endangered woody tree species. Information on seed viability in this species is lacking, it is clear that the seeds do not undergo maturation drying during the final phase of seed development and are thus shed in moist conditions as recalcitrant seeds are intolerant to both drying and low temperatures [11]. This plant species have high mortality in wild, and seeds are characterized by low percentage of germination was reported [7]. The present work for the first time, described a simple, rapid and reproducible method for enhanced *invitro* seed germination and development of *B. monosperma* var. *lutea,* from mature seed to establishment of seedlings in soil.

When conventional methods produce low or no germination*, in vitro* techniques can greatly enhance germination [1]. As *in vitro* techniques provide physical environment, mineral constituents strength of specific plant growth medium supplemented with both qualitative and quantitative PGRs are crucial for enhanced seed growth under *in vitro* conditions [2, 13]**.** *In vitro* seed germination and seedling growth is an important alternative to regenerate plants that are critically endangered and difficult to propagate due to in viable seed germination *via* conventional means [2, 14]. Therefore, in the present study two basic inorganic media in full or half strength (1/2) supplemented with various levels of PGR's (BA or TDZ alone), pre- hot water treatments at different time periods, were evaluated to achieve the aim of the present study.

Several investigations, showed BA in MS medium or TDZ in WPM are best formulations in inducing *in vitro* morphogenesis in wide variety of plant species [14, 15]. Albeit, reports available on their vice versa usage irrespective of plant nature [16, 17]. Such logic and vice versa was tested to find the best formulation for enhanced *in vitro* seed germination. Among the range evaluated, the basic MS full strength medium (MSF) with various levels of BA has worked out in inducing optimal *in vitro* seed germination percentage (20-65%). MSF supplemented with BAP (4.4µM) evoked superior seed germination response (65%), which was most effective in terms of shoot length (3.57 ± 0.06) as well as root length

(2.44 ± 0.17) of seedlings achieved after 3 weeks of culture.

Earlier studies have compared the effectiveness of TDZ and aminopurine cytokinins on seed germination at several concentrations [18, 19]. In the present study, TDZ could not elicit improved *in vitro* seed germination percentage as compared to that of BA. Media constituents supplemented with PGR's under half strength can induce elongated plantlets *in vitro* [20, 21]. Our finding has coincided with the previous reports in inducing seedlings with longest shoot and roots on MS(H) half strength medium containing BA. But here, half media constituents (either MS or WPM) failed to give optimal *in vitro* seed germination response as compared to MSF supplemented with BA.

Various time periods of exposure to per-hot water has played a remarkable effect on the induction on *in vitro* seed germination and development of seedlings with varying shoot and root lengths. Our results coincides with previous reports that seeds of specific plant species require specific time period of exposure to hot water for enhanced *in vitro* seed germination [22- 24].

In conclusion, the objective of the present work was to develop an enhanced *in vitro* seed germination, seedling development for *B. monosperma* var. *Lutea* . The methodology of pre-hot water treatment, type and strength of medium has successfully complimented enhanced *in vitro* seed germination and development protocol that is developed for the first time for this woody tree species. So the results presented demonstrate that mature seeds of *B. monosperma* var. *lutea* offer great potential for enhanced multiplication of this plant species *in vitro*. This protocol will strengthen large scale plantation activities towards conservation and restoration of this threatened and valuable *B. monosperma* var. *Lutea*.

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