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Riboflavin favors enhanced adventitious shoot formation in foliar explants of *Jatropha curcus* (L.)

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Abstract

Tender foliar explants of *Jatropha curcus* L. (~0.5 - 1.0 cm^2) were cultured in half strength MS medium supplemented with various combinations and concentrations of 6-benzylaminopurine (BAP), Kinetin (KIN), Naphthalene acetic acid (NAA) and Thidiazuron (TDZ) along with or without riboflavin. Morphologically distinct semi-friable and nodular type callus formations free of bud formation occurred in media devoid of riboflavin. Callus free adventitious buds with no or less callusing proliferated uniformly on the callus surface in media supplemented with concentrations of riboflavin (0.1, 0.3, 0.5, 1.0 mg.l^{-1}). Maximum of 16.3 ± 1.2 shoot buds and 14.5 ± 0.8 shoots were harvested in 4 weeks in medium supplemented with BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1}l and NAA 0.1 mg.l^{-1} together with 0.5 mg.l^{-1} riboflavin. Callus free bud shoot formation was distinct as 25.3 ± 1.2 buds and 20.7 ± 1.3 shoots were obtained from foliar explants after 6 weeks in media supplemented with this combination. Larger number of buds (38.2 ± 0.8) and shoots (34.1 ± 0.9) were obtained from callus cultured in media enriched with 0.1 mg/l TDZ and 0.5 mg/l riboflavin. The combination of 0.1 mg/l TDZ and 0.5 mg/l riboflavin gave the best regeneration for *J. curcas* leaf where maximum number of buds (45.6 ± 0.9) and shoots (43.2 ± 0.8) were harvested without intervening callus.

Keywords: 6-benzylaminopurine (BAP), callogenesis, caulogenesis, Thidiazuron (TDZ)

Introduction

Jatropha curcas L., the Barbados nut or Physic nut, is a perennial poisonous shrub of 5 m height belonging to the family Euphorbiaceae. Though it is an uncultivated non-food wild species, it is a potent plant of great economic value and all its parts have important pharmacological uses as enumerated in traditional Chinese medicine [1]. *J. curcas* is a fuel producing plant in the tropical and subtropical climate of India with oil content of 25-30% in the seeds and 50-60% in the kernel known as 'curcas oil'. The oil contains 21% saturated fatty acids and 79% unsaturated fatty acids. The oil is high in octane value and can be used directly in diesel engines, added to diesel fuel an extender or transesterized to a biodiesel fuel. The oil is clean as it reduces greenhouse gas emissions, has greater lubricity and reduces engine wear. As India has growing energy and transport fuel demand, *J.curcas* has potential to become one of world's key energy crops. India's vast agribiotechnological resources offer a clean substitute for expensive fossil fuel imports, thus enabling the country to meet the objectives of economic growth, fuel security and cleaner air. Certain drawbacks of the system are the seeds are heterozygous and the cuttings availability is seasonal, vegetative cuttings are easily uprooted as they do not form a tap root system [2].

Tissue culture of *Jatropha* species has been undertaken in different species. Morphogenesis from endosperm tissues has been reported [3]. High frequency regeneration is achieved in different explants of *J. integerrima* [4]. Few regeneration protocols have been reported for *J. curcas* using different explants like leaves [5, 6, 7, 8, 9,10], shoot tips [11], nodes and axillary nodes [6], petiole [5, 12], hypocotyls [5], cotyledons [12, 13, 14] etc., but multiplication rate was low for field applications. Moreover, no lab to land transfer protocol is available yet.

Tissue culture of *Jatropha* is an excellent avenue of research that leads to large scale multiplication of the plant. Hence it is of utmost importance to develop efficient regeneration protocols for *in vitro* culture of *Jatropha curcus*. The present work uses riboflavin, a water soluble vitamin which is involved in many cell growth and energy production processes. Riboflavin has been found to inhibit callus formation and may promote growth and quality of the shoots [15]. In the dark riboflavin stimulated rooting significantly in the presence of auxin (IBA), whereas rooting decreased when the vitamin was omitted and exposed to light [16]. Most of the studies carried out so far pertain to indirect organogenesis of callus, and very few reports are available on direct shoot regeneration, but the frequencies are reported to be low. The present work was carried out to investigate the effect of varying concentrations of riboflavin on multiple shoot induction by minimizing callus production. *In vitro* seedlings derived foliar explants were used as explants for the present study.

Materials and Methods

Seeds of *J. curcas* were obtained from Tamilnadu Agricultural University (TNAU), Coimbatore, Tamilnadu, India, and stored at room temperature prior to use. Seeds were soaked overnight in distilled water. Then, the seeds were surface sterilized with 90% ethanol for 30 sec. The seed coat was broken and embryos with cotyledon were aseptically inoculated on ½ MS medium. Leaf segments (~0.5 – 1.0 cm^2) were carefully excised from the *in vitro* seedlings of *Jatropha curcas* cultured on the above medium. Then the leaf explants were inoculated horizontally on the ½ MS medium supplemented with different concentrations and combinations of plant growth hormones *viz*., 6-Benzylaminopurine (BAP), Kinetin (KIN), Naphthalene acetic acid (NAA), and Thidiazuron (TDZ), in the presence or absence of riboflavin. All culture media were supplemented with sucrose 30 g.l^{-1} and solidified with 0.6% agar. The pH was adjusted to 5.8 ± 0.2. The cultures were incubated at 25ºC ± 2ºC under dark conditions for 3 weeks and followed by transfer into light (16 hrs light and 8hrs dark cycle; 38 - 40µ€ m^{-2}s^{-1}). Callus induction and shoot regeneration were recorded after 30 days. Each experiment was repeated at least three times with 100 explants. All data are presented as mean ± standard error. Data were analyzed with AGRES (Agricultural Research ANNOVA software - Version 7.01).

Results

In vitro seedlings were raised on ½ MS basal medium. Leaves from these seedlings were cultured on this medium supplemented with a combination of BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1} and NAA 0.1 mg.l^{-1} devoid of riboflavin. Solid callus was obtained within 15-20 days (Fig. 1a). Seedling leaf cultured in ½ MS medium fortified with TDZ 0.1 mg.l^{-1}, however developed into wavy callus with friable projections but devoid of organogenesis (Fig. 1b). When this callus was sub-cultured onto media fortified with the same hormonal strength along with riboflavin at different concentrations (0.1, 0.3, 0.5, 1.0 mg.l^{-1}), concentration dependent adventitious shoot bud formation was prominently visible. The callus slowly got fragmented and reduced in size while increasing the bud proliferation especially from the bottom of the agar medium and filling up the entire media surface (Fig. 1c & d). Chlorotic and necrotic symptoms appeared when the concentration of riboflavin exceeded 1.5 mg.l^{-1}. Of the various combinations tried, the optimum was augmented with BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1}, NAA 0.1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1}. At this combination, emergence of 16.3 ± 1.2 adventitious buds was noticed of which 14.5 ± 0.8 shoots of 0.8 - 1.5 cm length emerged after 3 weeks. Between hormones, TDZ proved to be better for inducing robust healthy shoots; maximum of 34.1 +0.9 shoots were obtained in ½ MS medium containing TDZ 0.1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1} (Table-1). When foliar explants were cultured in presence of riboflavin, multiple shoots proliferated without intervening callus. There was continuous addition of shoots to each cluster and the shoot clusters formed were practically free from remnants of callus. The shoot clusters differed in size each other and the most rapidly differentiating cluster consisted of 8 to 35 shoots varying in size but devoid of callus in each. Under optimal culture condition, maximum of 25.3 ± 1.2 buds and 20.7 ± 1.3 shoots were obtained from foliar explants after 6 weeks in medium supplemented with BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1}, NAA 0.1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1}. The effect of TDZ along with riboflavin on regeneration of *J. curcus* tender leaf disc was also studied. Maximum response was observed on ½ MS medium supplemented with TDZ 0.1 mg.l^{-1} in combination with riboflavin 0.5 mg.l^{-1}. The same combination proved best for multiplication of shoot buds and shoots recorded as 45.6 + 1.4 and 43.2 + 0.8 respectively (Table-2). In the absence of riboflavin, the callus formation as well as shoot formation were relatively lesser when foliar explants were subjected to TDZ treatment. It appeared that callus proliferation from foliar explants was prominent in media devoid of riboflavin and TDZ.

Discussion

Among many factors and cofactors that influence expression of genes in a plant, vitamins seldom get mentioned. In many cases, minute traceable quantities of vitamins are needed in vital metabolic processes of the body such as cell function, growth and energy production.

Vitamins are necessary compounds synthesized and utilized in plants. In tissue culture media, vitamin addition is not always common, since the amount needed by plants is relatively unknown and varies. Vitamins, in combination with other media constituents, have been shown to have direct and indirect effects on callus growth, somatic growth, rooting, and embryonic development [17]. Riboflavin (vitamin B_2) functions as part of several enzyme systems concerned with cellular respiration and oxidation of amino acids. Riboflavin acts as a precursor of FAD and FMN coenzymes, and nicotinic acid, pre- cursor of NAD and NADP, and participates in cellular redox reactions. It may have both beneficial and retarding effects.

Results presented in the present study reveal that riboflavin is greatly beneficial to minimize the callus proliferation phase simultaneously enhancing the regeneration potential of *J. curcas* foliar explants. Hitherto several important growth regulators and additives have been tested to contribute to *in vitro* regeneration of plants including *J. curcas*. Growth regulators are important factors which selectively influence the genes to trigger differentiation of cells in culture [18]. In media devoid of riboflavin, callus phase was found to be dominant and prolonged thereby greatly reducing the regenerative potential of the foliar explants. When riboflavin was added as an ingredient, the regenerative frequency was greatly enhanced as a result of minimal callus growth. The inhibitory effect of riboflavin on dedifferentiation of callus and regeneration of the different plant species was proved in *Carica papaya* (L.) [15]. In *in vitro* rooting of peach rootstock, the smallest concentration of 0.5 mg·l^{-1} of riboflavin caused average number and length of roots, whereas at 1.5 and 2 mg·l^{-1} of riboflavin chlorotic and necrotic symptoms appeared [19].

The results of the present investigation are unique in that riboflavin reduced the period of adventitious bud induction and further shoot elongation. The results in the present study confirm the efficiency of riboflavin on shoot regeneration from *in vitro* derived foliar explants by minimizing callus growth, which is evident from the large number of shoot buds (45.6 ± 0.9) formation and development into shoots (43.2 ± 0.8). The domination of callus phase was the mainly diminished shoot regeneration ability of *J. curcus* in the present study this was modified to have minimal callus growth and maximum production and harvest of shoots by the addition of riboflavin. The adventitious buds proliferated upon hypocotyls explants, and the bud forming frequency was approximately 21% [20]. Epicotyl explants yielded more adventitious buds (38%) than hypocotyls and differentiated shoots were developed from approximately 44% of calli much more than that of hypocotyl explants [21]. Leaf, petiole, cotyledon and hypocotyl explants easily gave shoot induction frequencies of approximately 44%-56% [22]. Embryogenic calli were obtained from leaf explants on MS basal medium supplemented with only 9.3 µM Kn. Induction of globular somatic embryos from 58% of the cultures was achieved on MS medium with different concentrations of 2.3–4.6 µM Kn and 0.5–4.9 µM IBA; 2.3 µM Kn and 1.0 µM IBA proved to be the most effective combination for somatic embryo induction in *J. curcas* [23]. Investigations involved in *in vitro* cultures were minimal or moderate in most of the shoot proliferation studies of *J. curcus*, where the relative level of direct shoot regeneration was reported as low. In the foliar culture of the present study, however more than 90% of the cultures supplemented with riboflavin responded for shoot production very optimally. What was a haphazard nature of massive callus proliferation was fine-tuned by the presence of riboflavin to yield minimum callusing and dedicated production of vegetative buds. There was domination of exclusive callus phase reducing the shoot forming ability of foliar explants in media devoid of riboflavin. Addition of riboflavin promoted remarkable bud and shoot formations without waste of available tissue and time contributing to improved shoot harvest every time. Minimizing callus formation and maximizing budding is in fact a challenge; this was circumvented and successfully changed for the better in this system. As a result, an efficient protocol was developed with minimal dose of riboflavin and metabolic alteration to obtain exclusive shoot production with TDZ as a growth regulator of choice.

Table 1: Influence of selected concentrations of BAP, NIN, NAA and TDZ and varied concentrations of riboflavin on shoot bud and shoot development in foliar callus of *J.curcas* cultured in half strength MS medium. Note the increasing number of shoots harvested from media containing riboflavin. Mean values within the same column followed by the same superscript(s) do not significantly differ (P<0.05) according to ANOVA and LSD *multiple* range test.

S. No.	Concentration of Plant Growth Regulators (mg.l^{-1})				Concentration of riboflavin (mg.l^{-1})	No. of average shoot buds/explant	No. of average shoots/explant
	BAP	KIN	NAA	TDZ			
1.	2.0	0.2	0.1	-	0.1	11.7 ± 1.3^{fg}	10.3 ± 0.9^{fg}
2.	2.0	0.2	0.1	-	0.3	13.6 ± 0.9^{f}	11.6 ± 1.1^{efg}
3.	2.0	0.2	0.1	-	0.5	16.3 ± 1.2^{e}	14.5 ± 0.8^{e}
4.	2.0	0.2	0.1	-	1.0	14.8 ± 1.1^{ef}	12.7 ± 0.9^{ef}
5.	-	-	-	0.1	0.1	25.8 ± 1.2^{d}	21.7 ± 1.3^{d}
6.	-	-	-	0.1	0.3	31.4 ± 1.2^{c}	28.6 ± 1.2^{c}
7.	-	-	-	0.1	0.5	38.2 ± 0.8^{a}	34.1 ± 0.9^{a}
8.	-	-	-	0.1	1.0	35.1 ± 0.9^{ab}	30.7 ± 0.8^{bc}

Table 2. Influence of selected concentrations of BAP, KIN and NAA with the same concentration of TDZ (0.1 mg.l^{-1}) and varied concentrations of riboflavin on bud and shoot formations in foliar explants cultures of *J. curcas*. Note the increased number of shoots (43.2 + 0.8) harvested from a combination of TDZ 0.1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1}. Mean values within the same column followed by the same superscript(s) do not significantly differ (P<0.05) according to ANOVA and LSD multiple range test.

S. No.	Concentration of Plant Growth Regulators (mg.l^{-1})				Concentration of riboflavin (mg.l^{-1})	No. of average shoot buds/explant	No. of average shoots/explant
	BAP	KIN	NAA	TDZ			
1.	2.0	0.2	0.1	-	0.1	19.7 ± 1.3^{fg}	14.1 ± 0.9^{g}
2.	2.0	0.2	0.1	-	0.3	21.2 ± 1.4^{fg}	17.7 ± 0.8^{fg}
3.	2.0	0.2	0.1	-	0.5	25.3 ± 1.2^{e}	20.7 ± 1.3^{e}
4.	2.0	0.2	0.1	-	1.0	23.9 ± 1.1^{ef}	18.2 ± 0.8^{ef}
5.	-	-	-	0.1	0.1	31.3 ± 0.9^{d}	29.4 ± 1.1^{d}
6.	-	-	-	0.1	0.3	36.7 ± 0.8^{c}	34.8 ± 1.2^{c}
7.	-	-	-	0.1	0.5	45.6 ± 0.9^{a}	43.2 ± 0.8^{a}
8.	-	-	-	0.1	1.0	42.8 ± 1.2^{ab}	40.5 ± 1.0^{b}

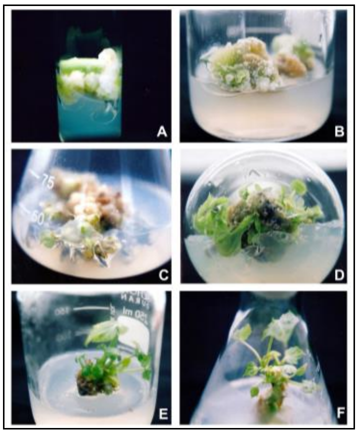

Fig 1: [A] Callogenesis in foliar explants of *J.curcas* cultured on ½ MS medium fortified with BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1}, NAA 0.1 mg.l^{-1} and without riboflavin. [B] Calogenesis in foliar explants cultured on ½ MS medium fortified with TDZ 0.1 mg.l^{-1} and without riboflavin [C] Multiple shoot buds derived from callus re-cultured on ½ MS medium fortified with BAP 2.0 mg.l^{-1}, KIN 0.2 mg.l^{-1}, NAA 0.1 mg.l^{-1} and with riboflavin 0.5 mg.l^{-1}. [D] Multiple shoots obtained in re-cultured callus in ½ MS medium fortified with a combination of TDZ 0.1 and riboflavin 0.5 mg.l^{-1}. [E] Buds proliferated upon foliar explants cultured for 6 weeks using TDZ 0.1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1}. [F] Mature shoots of *J. curcas* differentiated on TDZ 0. 1 mg.l^{-1} and riboflavin 0.5 mg.l^{-1} after 8 weeks of culture.

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