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Protocol for augmented shoot organogenesis in selected variety of soybean [*Glycine max* L. (Merr.)]

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Development of a reproducible, versatile and efficient *in vitro* plant regeneration system is highly warranted for Indian soybean varieties for their mass multiplication in view of their commercial significance. Accordingly a protocol for direct shoot organogenesis in soybean variety JS 335 has been developed. Using cotyledonary node explants significant organogenic responses, mean shoot number and shoot length were observed when these were incubated on MS medium supplemented with 0.89 µ*M* Benzyladenine (BA) and 5 µg/L triacontanol (TRIA) where in 9.3 ± 0.5 shoots were obtained. TRIA at 5 µg /L able to produce 6.8 ± 0.5 shoot buds in presence of 0.98 µ*M* IBA and 0.89 µ*M* BA. Highest mean shoot buds (14.0 ± 0.5 and 9.0 ± 0.5) and mean shoot length (4.6 ± 0.3 and 10.0 ± 0.7) were obtained when cotyledonary node and shoot tip explants were cultured on MS medium containing 0.14 µ*M* gibberellic acid (GA_3), 0.89 µ*M* BA and 5 µg/L TRIA. Moreover, TRIA supported highest mean root number (6.3±0.5) and root length (21.5 ± 0.57 cm). Field survival of *in vitro* derived plants of TRIA treatment was 70% and the overall growth and seed yield was also significantly better than control plants. This protocol may be used for improving the *in vitro* regeneration of soybean variety JS 335 for transformation studies.

Keywords: Cotyledonary node, *Glycine max, In vitro* rooting, Microshoots, Shoot buds, Triacontanol

Soybean [*Glycine max* (L.) Merr.], a rich source of food protein is grown on more areas worldwide than any other pulse crop. In India the area under soybean cultivation is on the rise and there is a huge demand for soy oil and other soy based products. Apart from its rich protein and oil content, soybean contains beneficial secondary metabolites such as isoflavones, phenolic compounds, saponins and phytic acids[1]. Hence soybean receives great importance due to its nutraceutical values and for its application in the area of functional foods. In attaining this goal, we owe much to the biotechnological techniques for producing new breeding materials that may not be available in the germplasm.

Triacontanol (TRIA) (myricyl alcohol), a long chain 30-carbon primary alcohol, is a naturally occurring plant growth promoter[2,3] first identified in wax of alfalfa[4]. The growth inducing property of TRIA was demonstrated in many plants for increased crop yield[5], improved physiological response[6] and enhanced growth and yield parameters[7]. The efficacy of TRIA on *in vitro* propagation of *Decalepis hamiltoni*[8], induction of early bolting in *Arabidopsis thaliana*[9], enhanced somatic embryogenesis in Coffee[10] and multiple shoot induction along with improved annatto pigment yield of *Bixa orellana*[11] was documented. TRIA was also found to enhance the production of secondary metabolites in *Artemisia annua*[12], *Decalepis hamiltonii*[13] and *Salvia officinalis*[14]. Pertaining to soybean, n-triacontanol was able to restore the normal metabolic process in the salt stressed soybean[15].

Various biotechnological methods for soybean have been developed for its micropropagation, callus mediated regeneration[16], direct organogenesis[17,18] and somatic embryogenesis[19,20]. But most of these methods are specific for certain varieties and breeding lines. Improving the *in vitro* shoot multiplication of soybean under the influence of TRIA will have an advantage. It is of interest to understand the effect of TRIA in augmented plant growth and seed yield. In the present communication, we report an improved *in vitro* multiplication of soybean under the influence of TRIA.

Materials and Methods

Source of plant material—Soybean seeds of JS 335 genotype were procured from Gandhi Krishi Vigyana

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Kendra, University of Agricultural Sciences, Bangalore, India. Uniform size soybean seeds were handpicked and surface sterilized in 70% ethanol for 30 seconds followed by 0.2% carbendazium for 5 min and 0.1% mercuric chloride (w/v) for 1 min. The surface sterilized seeds were rinsed thrice in sterile distilled water and cultured in tissue cultured bottles, each containing 40 mL MS basal medium[21] for germination. The *p*H of the medium was adjusted to 5.8 then autoclaved at 121ºC for 20 min. The cultures were maintained at a light intensity of 57 µmol m^{-2} s^{-1} (illumination supplied by cool-white fluorescent tubes) with 16-h photoperiod at 25 ±2°C. Twelve days after seed germination, cotyledonary nodes and shoot tips (~1 cm long) were excised from the seedlings.

Triacontanol stock solution—Triacontanol [CH_3 $(CH_2)_{28}$ CH_2OH] stock solution was obtained from Sigma-Aldrich Chemicals (Bangalore) and prepared by dissolving 1 mg of TRIA in 0.75 mL of $CHCl_3$ containing one drop of Tween 20 and was gradually diluted with distilled H_2O to a final volume of 200 mL[10].

Effect of TRIA on in vitro multiple shoot formation—To induce multiple shoots, varying concentrations of TRIA (0, 2, 5 and 10 µg/L) was added to the multiple shoot induction media containing MS basal salts and vitamins with 30 g/L sucrose (w/v), 8.0 g/L (w/v) agar, 0.89 µ*M* benzyladenine (BA) and 0.98 µ*M* indole-3-butyric acid (IBA). Single standardized concentration of BAP, IBA and GA_3 which had shown best results previously[22] were selected and used in the present experiment. Cotyledonary node and shoot tip explants were inoculated onto this medium and cultured for 4 weeks under 16:8 h photoperiod. Shoot buds produced were sub cultured onto shoot bud elongation medium containing MS basal salts and vitamins with 0.14 µ*M* gibberellic acid (GA_3), 0.89 µ*M* BA and varying concentrations of TRIA (2, 5 and 10 µg/L). After 4-6 weeks of culture, the number of elongated shoots, shoots length, node number and fresh weight of shoots were determined. Elongated shoots (~ 3cm) were further transferred to rooting medium containing MS salts and vitamins with 2-10 µg/L TRIA and cultured for 4 weeks. The rooted plantlets were removed from the medium, washed in running tap water and planted in micro-pots containing sand:compost mixture (1:2) and were maintained under greenhouse for a month and then transplanted to micropots for further studies to document growth parameters. One month old micropropagated plants were transplanted to individual pots containing soil:sand:farm yard manure in the ratio of 2:1:1 and allowed to grow for one more month. Similarly in order to maintain control plants, one-month-old seedling plants were transplanted in individual pots. Potted plants were irrigated on alternate days for three months. Various parameters such as plant height, number of flowers, number of pods, number of seeds per plant, and seed weight were documented.

Statistical analysis—Each experiment was repeated thrice with 20 replicates. To evaluate the growth performance 10 plants each of control (seedling plants) and micropropagated hardened plants were used for the study and the experiment was repeated twice. Data were analyzed statistically by analysis of variance (ANOVA), and the difference between the means of sample was analyzed by the least significant difference (LSD) test at a probability level of 0.05.

Results

Effect of TRIA was evident in both the multiplication and rooting phase of soybean *in vitro* organogenesis. In the multiplication phase, maximum response was obtained in the medium containing 0.89 µ*M* BA, 5 µg/L TRIA for cotyledonary node (Table 1; Fig. 1), and the combination of 0.89 µ*M* BA, 0.98 µ*M* of IBA and 5 µg/L TRIA for shoot tip explants (Fig. 2). In the absence of TRIA the response for shoot bud proliferation was poor on medium containing either BA alone or in combination of 0.89 µ*M* BA, 0.98 µ*M* of IBA. This was evident in both types of explants. Even the lowest concentration of TRIA (2 µg/L) incorporation to the medium comprising 0.89 µ*M* BA resulted in an increase in the number of shoots (8.5 ± 0.7 and 3.0 ± 1.0) and shoot length (1.33 ± 0.5 and 1.62 ± 0.6 cm) respectively for cotyledonary node and shoot tip explants. Overall significant increase in the mean shoots number and shoot length was observed at lower concentrations of TRIA (2, 5 µg/L), whereas higher TRIA concentration (10 µg/L) did not show any significant difference compared to the control (Table 1). TRIA at 5 µg/L along with 0.89 µ*M* BA recorded the maximum number of shoots (9.3 ± 0.5 and 5.3± 0.5) and shoot length (2.33 ± 0.5 and 4.16 ± 0.7 cm) for cotyledonary node (Fig. 1 a) and shoot tip (Fig. 2a) explants respectively (Table 1). A similar trend was evident in presence of 0.89 µ*M* BA, 0.98 µ*M* IBA and 5 µg/L TRIA. However, addition of 0.98 µ*M* IBA to the tested concentrations of TRIA reduced shoot number (7.0 ± 0.5) and increased shoot length (3.83 ± 0.7 cm) in case of cotyledonary node explants whereas, it significantly increased the number of shoots (6.8 ± 0.5) and shoot length (4.5 ± 0.5 cm) in case of shoot tip explants. Apart from this, small shoot buds were produced from both cotyledonary node and shoot tip explants along with light greenish friable callus at all the treatments of TRIA and it was more at 5 µg l^{-1} TRIA treatment (12-15 shoot buds) followed by 2 and 10 µg/L treatment respectively. But callusing was more in the presence of 10 µg/L TRIA.

These shoot bud clumps upon transferring to elongation medium comprising of 0.89 µ*M* BA, 0.14 µ*M* GA_3 and 2-10 µg/L TRIA resulted in elongation of shoot buds into micro shoots, with 90% response for cotyledonary node and 80% for shoot tip explants respectively, in the presence of 5 µg/L TRIA (Table 2). Both the explants showed significant difference for number of shoots and shoot length for the TRIA concentrations compared with the control. Cotyledonary nodes recorded a maximum of 14 ± 0.5 shoots/explant with an average shoot length of 4.6 ± 0.3 cm (Fig. 1b), whereas shoot tip explants recorded an

Table 1—Induction of multiple shoots from cotyledonary node and shoot tip explants of soybean in the presence of BA, IBA and TRIA [*Values are mean ± SD of 20 explants]

Explant	MSB+BA (µ*M*)	TRIA (µg/L)	IBA (µ*M*)	*No. of explants producing shoots (%)	No. of shoots/ (explants)	*length of shoot (cm/ explant)
Cotyledonary node	0.89	-	-	50.0^{d}	4.0 ± 0.7^{cd}	0.60 ± 0.5^{d}
	0.89	2	-	75.0^{bc}	8.5 ± 0.7^{ab}	1.33 ± 0.5^{cd}
	0.89	5	-	100.0^{a}	9.3 ± 0.5^{a}	2.33 ± 0.5^{bc}
	0.89	10	-	83.3^{b}	6.3 ± 0.7^{c}	2.16 ± 0.2^{bc}
	0.89	2	0.98	75.0^{bc}	4.3 ± 1.2^{cd}	1.83 ± 0.7^{c}
	0.89	5	0.98	83.3^{b}	7.0 ± 0.5^{b}	3.83 ± 0.7^{a}
	0.89	10	0.98	100.0^{a}	3.5 ± 0.7^{cd}	2.50 ± 0.3^{bc}
Shoot tip	0.89	-	-	30.0^{d}	2.0 ± 1.0^{d}	0.80 ± 0.4^{cd}
	0.89	2	-	50.0^{c}	3.0 ± 1.0^{cd}	1.62 ± 0.6^{c}
	0.89	5	-	80.0^{a}	5.3 ± 0.5^{b}	4.16 ± 0.7^{a}
	0.89	10	-	60.0^{b}	3.6 ± 0.5^{c}	2.75 ± 0.3^{b}
	0.89	2	0.98	40.0^{cd}	3.3 ± 1.2^{c}	3.50 ± 0.5^{ab}
	0.89	5	0.98	66.6^{b}	6.8 ± 0.5^{a}	4.50 ± 0.5^{a}
	0.89	10	0.98	50.0^{c}	2.7 ± 0.7^{cd}	1.25 ± 0.3^{cd}

Medium without growth regulators did not support shoot proliferation.
Mean values with different alphabets are statistically significant at $P< 0.05$. Data were recorded after 4 weeks in the triacontanol media

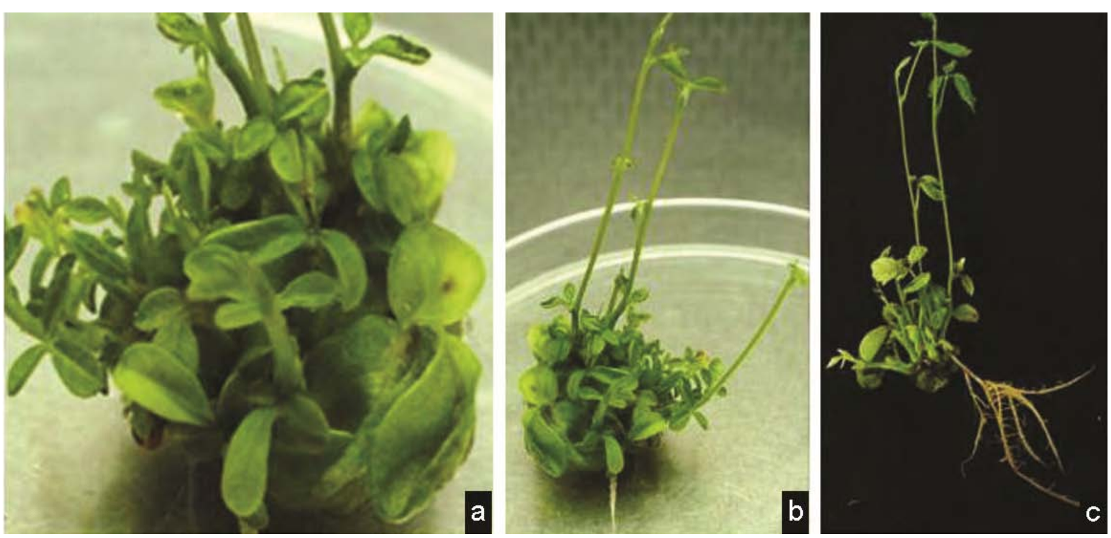

Fig. 1—Effect of TRIA on *in vitro* shoot regeneration of soybean (cv. JS 335) from cotyledonary node explants on MS medium: (a)-induction of multiple shoots in presence of 0.89 µ*M* BA and 5.0 µg/L TRIA; (b)-shoot elongation and emergence of new shoots in presence of 0.14 µ*M* GA_3, 0.89 µ*M* BA and 5.0 µg/L TRIA; (c)-*in vitro* rooting of plantlets on medium containing 5.0 µg/L TRIA.

average of 9.0 ± 0.5 shoots/explant with an average length of 10.0 ± 0.7 cm (Fig. 2b, c). The shoot length almost doubled with the addition of GA_3 along with TRIA and BA in case of cotyledonary nodal explants, whereas the shoot tips exhibited a three fold increase in shoot length, compared to the control (0.89 µ*M* BA and 0.14 µ*M* GA_3). Elongation medium devoid of BA or GA_3 did not show any response, however elongated shoots were obtained when TRIA alone at 5 µg/L was added to the medium (Fig. 2c).

Efficient response for root number, root length, number of nodes, shoot length and fresh weight of shoots were evident during root induction phase in the presence of TRIA after 4 weeks of culturing (Fig. 1d, Table 3). Number of roots and root length considerably increased with 2 and 5 µg/L TRIA and decreased at higher concentration of 10 µg/L. Although TRIA was found to be less effective at higher concentrations (5 µg/L and 10 µg/L), it induced more number of roots (6.3 ± 0.5 and 5.5 ± 0.7) with increased root length (21.5 ± 0.57 cm and 17 ± 0.6 cm) (Table 3) respectively than the control (Table 3). Apart from this, TRIA positively influenced shoot growth and increased the number of nodes, with reduced length of internodes during this root induction phase (Table 3). The maximum shoot length was recorded at 5 µg/L concentration (8.0 ± 0.63), whereas the highest number of nodes was found at 10 µg/L (5 ± 0.56) with slightly reduced shoot length. Fresh weight of the plantlets was significantly higher at all concentrations of TRIA than that of the control. Fresh weight of plants were highest (6.2 ± 0.4) in the presence of medium containing 2 µg/L TRIA and 10 µg/L TRIA (Table 4). The growth parameters such as shoot length, number of flowers, pods and seeds along with average weight of seed were recorded in both micropropagated and seedling derived plantlets (Table 4). Results of various parameters showed that micropropagated plants outperformed than seedling based plants (Table 4). There was a 16%, 24% and 16% improvement in number of flowers, number of pods and seed yield in micropropagation derived plant compared to seedling derived plants respectively.

Discussion

On the basis of the response in presence of TRIA obtained for soybean variety JS 335 in this study, the same has been advocated against another soybean variety Hardee to find out influence of TRIA on shoot multiplication. According to our earlier communication on *in vitro* shoot multiplication for

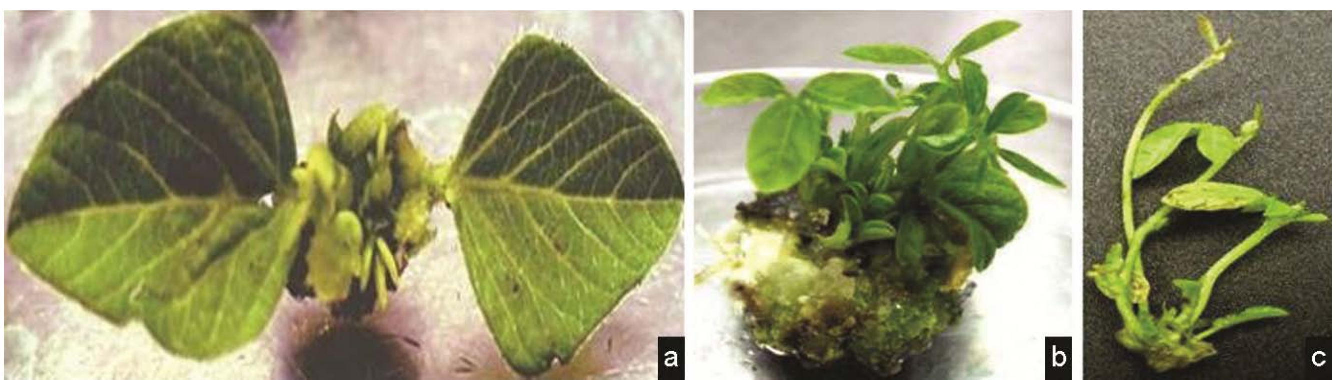

Fig. 2—Effect of TRIA on *in vitro* shoot regeneration of shoot tip explants of soybean cv. JS 335 on MS medium: (a)-multiple shoot induction in presence of 0.89 µ*M* BA and 5.0 µg/L TRIA; (b)-shoot elongation and new shoots emergence in presence of 0.98 µ*M* IBA, 5.0 µg/L TRIA and 0.14 µ*M* GA_3; (c)-elongated soybean shoots in MS medium with 5.0 µg/L TRIA.

Table 2—Effect of TRIA on shoot bud elongation on MS basal medium comprising 0.14 µ*M* GA_3 and 0.89 µ*M* BA
[*Values are mean ± SD of 20 explants]

TRIA (µg/L)	No. of explants producing shoots (%)		*No. of shoots /explants emerged		*Length of shoot (cm/explant)	
	Cotyledonary node explant	Shoot tip explant	Cotyledonary node explant	Shoot tip explant	Cotyledonary node explant	Shoot tip explant
-	30^{d}	40^{cd}	4.2 ± 0.7^{d}	6.2 ± 0.7^{cd}	2.0 ± 0.5^{d}	3.0 ± 0.5^{cd}
2	75^{ab}	54^{bc}	9.3 ± 1.2^{bc}	7.3 ± 1.2^{c}	3.2 ± 0.3^{cd}	6.3 ± 0.7^{bc}
5	90^{a}	80^{ab}	14.0 ± 0.5^{a}	9.0 ± 0.5^{bc}	4.6 ± 0.3^{c}	10.0 ± 0.7^{a}
10	65^{b}	50^{c}	8.5 ± 0.7^{bc}	6.5 ± 0.7^{d}	2.5 ± 0.5^{d}	4.5 ± 0.3^{c}

Mean values with different alphabets are significantly different at $P < 0.05$. Data were recorded after 4 weeks in the triacontanol media.

soybean Hardee variety[22] a maximum of 6-8 shoots per nodal explant were obtained in presence of optimized growth regulators. But in presence of TRIA this Hardee variety too showed enhanced shoot multiplication wherein up to 12 shoots were obtained. As the response for this variety was similar to that of JS 335 variety, the detailed data has not been given. This indicates the suitability and efficiency of TRIA on *in vitro* shoot multiplication in soybean.

Induction of multiple shoots from both shoot tip and cotyledonary leaf nodes in the presence of BA is evident in our study and similar observations were noticed in other cultivars of soybean[23,22] and also in other plants[24,25]. In our earlier publication[22], we reported that explants grown in the medium containing BA and GA_3 alone couldn't show much response. Besides, cotyledonary node explants of cv. Hardee grown in media containing TDZ showed a maximum of 8 shoot buds per explants. We have used selected variety of soybean JS335 for our study and the response for multiple shoots was good (14 shoots/explants) compared to earlier reports[22]. The response is genotype specific, which was well established phenomenon in various plants and the data obtained in the present study is comparable to less than the international varieties[22].

Table 3—Effect of TRIA added during the root induction phase on number of nodes, shoot length and fresh weight, number of roots and root length of soybean*
[Values are mean ± SE of 20 explants]

TRIA (µg/L)	No. of nodes	Shoot length (cm)	Fresh weight (gm)	Root number	Root length (cm)
-	2.3^{c}	4.0^{d}	3.1^{d}	1.8^{c}	5.1^{d}
2	4.3^{b}	5.4^{b}	5.8^{b}	4.3^{b}	10.8^{c}
5	5.6^{a}	7.8^{a}	6.2^{a}	6.3^{a}	21.5^{a}
10	5.0^{ab}	4.7^{c}	4.3^{c}	5.3^{ab}	17.6^{b}

*Values with different alphabets are significantly different at $P < 0.05$. Data were recorded after 4 weeks in the triacontanol media.

Table 4—Comparison of field grown micropropagated and seedling derived soybean plants
[Values are mean ± SE of 10 seedlings]

Parameters*	Micropropagated derived plant	Seedling derived plant
Shoot length (cm)	91.0 ± 0.71	78.5 ± 0.66
Number of flowers	106.0 ± 0.55	88.0 ± 0.41
Number of pods	95.0 ± 0.62	72.0 ± 0.53
Number of seeds/plant	250.5 ± 0.93	214.5 ± 0.83
Weight of single seed (g)/seed	0.153 ± 0.16	0.123 ± 0.18

*Most parameters were measured 60 d after anthesis.

Influence of TRIA on proliferation of shoot buds from shoot tip and cotyledonary leaf node explants was found to be synergistic with BA and IBA in this study and was supported by similar observations in other plants[8,11]. Combination of BA and IBA or shoot bud proliferation was also well documented in case of sugar beet[26]. TRIA alone also induce shoot buds from shoot tip explants as reported in *Dendrobium nobile*[27]. At low concentrations, TRIA promoted both shoot growth and rooting of plants, but at high concentrations it had a slight inhibitory effect. This may be due to the sensitivity of whole explants to extremely low doses of TRIA[28]. Corresponding data published in the literature also indicate that TRIA has an inhibitory effect above certain concentrations[8,29]. Cotyledonary node explants on medium containing 5 µg/L TRIA had the most number of shoots with maximum shoot length. TRIA enhanced the shoot length and number of nodes with a reduced length of internodes. This response of TRIA could be due to various reasons as reported earlier and not simply caused by water uptake and cell enlargement but rather by an increase in cell number[30]. After initial application of TRIA, a metabolite of TRIA or a secondary messenger moves rapidly in plants and influences enzymes relating to carbohydrate metabolism[3] and growth processes[31], which might be responsible for the high growth activity in plants. Moreover, the response is concentration dependent. In case of *Melissa officinalis*[29] and *Capsicum frutescens*[8] the optimum level of TRIA required for shoot multiplication was 5 µg/L, however, 10-20 µg/L TRIA was required to induce shoot buds in *Thymus mastichina*[32]. The inter nodal length of *in vitro* shoots was less compared to controls under 10 µg/L of TRIA treatment in our study. This is in contrast to early bolting effect of 0.1 to 0.6 µ*M* TRIA as shown in *Arabidopsis thaliana*[9]. Supportive role of TRIA on *in vitro* shoot multiplication and antioxidant compounds such as carnosic acid and diterpenoids in shoot cultures of *Salvia officinalis*[14] was well demonstrated. An important observation in our study is an increase in number of flowers in TRIA induced micropropagated *ex vitro* grown plants which was substantiated by the supportive role of TRIA in inducing the quality and yield of flowers as in the case of *Chrysanthemum morifolium and* orchids[33,34]. The present work shows the stimulating effect of TRIA on shoot multiplication and rooting at very low concentrations, which clearly indicates that TRIA can be effectively used as a growth regulator for *in vitro* regeneration of soybean.

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