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ASSESSING THE EFFECT OF PHYTOHORMONE ON GROWTH AND GERMINATION OF SOYBEAN [*GLYCINE MAX* (L.) Merr.] FROM COTYLEDONARY NODE

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Abstract

Glycine max (L.) Merr. production is affected by a number of biotic and abiotic stresses. In order to overcome these challenges, development of transgenic plants, production of high-quality varieties and secondary metabolites, preservation of rare plants, and protection of germplasms is of prime importance. In this regard, proper regeneration system for plants growth is needed to overcome the dormancy and low germination issues by using appropriate growth hormones. Therefore, we analyzed the effect of various phytohormones 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-benzyladenine (BAP), Kinetin (KIN) and Indole-3-butryic acid (IBA) on *Glycine max* growth to investigate the influences of explants types. Results revealed that good callus is produced from cotyledonary node on 2, 4-D (4.0 mg L^{-1}). The highest percentage of regenerated shoots (88%) was found on BAP+KIN (2.0+1.0 mg L^{-1}) with maximum shoots number (3.00±0.09) and maximum length of shoot is (4.22±0.08) cm. The IBA (0.5 mg L^{-1}) is excellent for root formation. Here, applied procedure used for further development of regeneration and transformation efficiency of *Glycine max*.

Key words: Economical approach, Marginal lands, Salt tolerance, Seed pre-treatment

Introduction

Glycine max seeds contain high quality proteins and oil with unsaturated fatty acid (85%) making it most important worldwide (Yaklich *et al.,* 2002). Due to its nutritional and medicinal importance, it has been widely grown for various uses (Isler & Vural, 2010). In the era of climate change, *Glycine max* productivity is hampered due to biotic or abiotic stresses leading to reduced yields and poor seed quality (Phat *et al.,* 2015). In this regard, development of stress tolerant plants using *In vitro* selection and genetic engineering is important biotechnological tool to address this issue (Sojkova *et al.,* 2016). Among them, *In vitro* selection emerged as feasible and cost effective way within limited space and time (Pathak *et al.,* 2017). Regarding explants for callus culture establishment, different parts of soybean plants are being used such as., seedling shoot tips (Kartha *et al.,* 1981), hypocotyls (Yoshida, 2002; Wang & Xu, 2008), cotyledonary nodes (Liu *et al.,* 2010), cotyledons (Joyner *et al.,* 2010) and leaves (Wright *et al.,* 1987. Among them, most efficient and totipotent explants type is cotyledonary node with great regeneration effeiciency monitored by different plant growth regulators for production of soybean transformed plants (Olhoft *et al.,* 2003).

However, problems associated with low transformation efficiency, slow regeneration process need to resolve through altered micro climates (phytohormone concentration, explants, media types and genotypes (Phat *et al.,* 2015). Different concentrations and types of hormones play key role in differentiation, and morphogenesis (Kim *et al.,* 2009). An active and fast regeneration procedure is required that may regenerate more plantlets in a short time period. Therefore, objective of this study was to evaluate the various phytohormones and their concentrations used for *Glycine max* seedling establishment in order to develop the conducive condition for growth and provide better platform for genetic engineering.

Materials and Methods

Seed source and sterilization procedure: Mature seeds of *Glycine max* L. (Variety Swat-84) were washed with running tap water for at least 30 min for surface sterilization. After washing, seeds were treated with a solution Tween 20 (5% v/v) for at least 10 min and then sterilized with 70% alcohol for 1 min and finally surface Sterilized with mercuric chloride (0.1% w/v) for at least 5 min, after that the seeds were washed three times with autoclaved distilled H_20 in the laminar air flow chamber.

Basal media, culture environment: Murashige & Skoog (MS) medium, salts, vitamins, 3% sucrose, 2.5 (g) phytagel and different hormone (growth regulators) were used for regeneration of plants. The pH was adjusted to 5.7-5.8. MS media autoclaved at 17 psi for twenty min then cool down (Murashige & Skoog, 1962). Media were solidified at room temperature. The cultured bottles were shift and placed in controlled growth room.

Inoculation: *Glycine max* seeds were inoculated on the MS media under sterilized condition in a laminar air flow chamber. Four seeds per bottles were inoculated perpendicularly above the surface of the media.The seeds were kept at 25 ± 2°C in the dark for the first 2 days and then transferred to a 16 h photoperiod and 8 hours dark cycle.

Callus induction and maintenance: Explants (Cotyledonary node and leaves) were taken from 14 days old seedlings, and inoculated on MS medium with various concentrations of phytohormones for induction of callus for 10 to 15 days. The concentrations used for callus induction were as follows; 2,4-D (2,4-dichloro phenoxy acetic acid) (0.5, 1.5, 2.0, 2.5, 3.0, 4.0, 4.5 mg L^{-1}) solitary, different combinations were also used Kin

(kinetin (0.2, 0.5, 1.0, 2.0, 2.5 mg L^{-1}) along with 2,4-D (1.0, 2.0 mg L^{-1}) and BAP (6-benzylamino purine) + NAA (*Naphthalene acetic acid* (0.1, 0.4, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0 mg L^{-1}). The time taken for Callus induction, callus weight and the effect of growth regulators on callus formation capacity was recorded.

Shoot and root formation: Callus was then transferred to shoot induction medium for 2-4 weeks and then number and length of shoot was recorded and % response was calculated. After establishment of shoots, shootlets were transferred to rooting medium. The shoot induction growth regulators were used as follows; BAP (6-benzylamino purine) (0.5,1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg L^{-1}) and KIN (1.0, 2.0, 3.0 mg L^{-1}) alone, and in combination of BAP+ NAA (0.4, 1.0, 1.5, 2.0 mg L^{-1}) and BAP (1.0, 2.0, 4.0 mg L^{-1}) + KIN (1.0 mg L^{-1}). The root formation growth regulators were as follows; IBA (Indole-3-butyric acid) IAA (Indole-3- acetic acid) NAA (Naphthalene acetic acid*)* (0.5, 1.0, 1.5, 2.0 mg L^{-1}).

Statistical analysis

All data obtained from experiment was statistically analyzed according to mean values analyzed variance (ANOVA) through SPSS software and the comparison of mean was obtained using Duncan's Test. All the analysis was recorded as significant at $p \leq 0.05$.

Results

Callus formation: The cotyledonary node and leaves of the plant were selected as explants for callus induction on MS medium with or without growth regulator (Fig. 1a, 1b, 1c). The callus established from the explants was of yellow colour. As evident from callus weight at cotyledonary node (0.17 g) and leaves (0.16 g) respectively in basal media (without any hormone) showed minimum callus induction. While varying concentration of 2, 4-D hormone in MS medium (0.5-4.5 mg L^{-1}) triggered callus proliferation in both cotyledonary node and leaves. 2, 4-D (4.0 mg L^{-1}) concentration showed the maximum weight of callus from cotyledonary node (0.8g) and leaves (0.73g). In this experiment, 2, 4-D alone was efficient in initiating callus from the cotyledonary node at all concentrations ranging between 0.5-4.5(mg L^{-1}) showing statistically significance (for explants (E) and their interaction (C×E). Induction and proliferation of callus was observed with other growth hormone combinations such as BAP and NAA in different concentrations (0.4, 0.5, 1.5, 2.0, 3.0 mg L^{-1}). The highest callus weight was observed at concentration of 2 and 3.0 mg L^{-1} both on cotyledonary node and leaves, while other hormone concentrations induced low amount of callus (Fig. 1b). whereas, cotyledonary node explants also exhibited good response by high amount of callus with supplementation of KIN+ 2, 4-D (0.5+1.0, 0.5+2.0, 2.5+1.0 mg L^{-1}) (Fig. 1c). Statistically significant differences ($p<0.05$) were also found between explants, concentrations and their interaction (C×E) for this hormone combination (Fig. 1c)

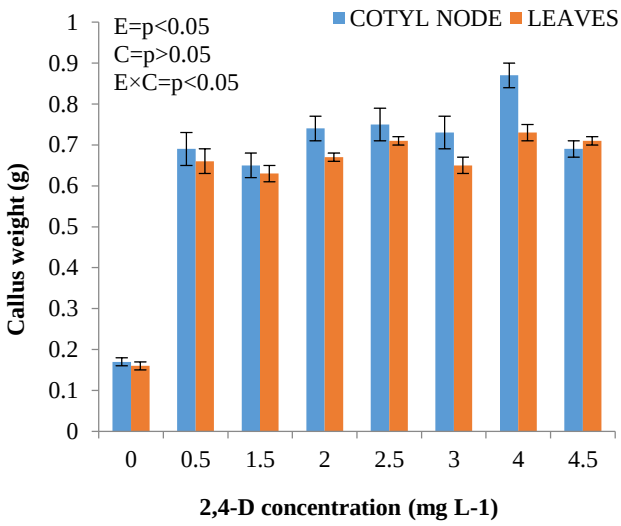

Fig. 1a. Callus induction in *Glycine max* on concentrations of 2, 4- D.

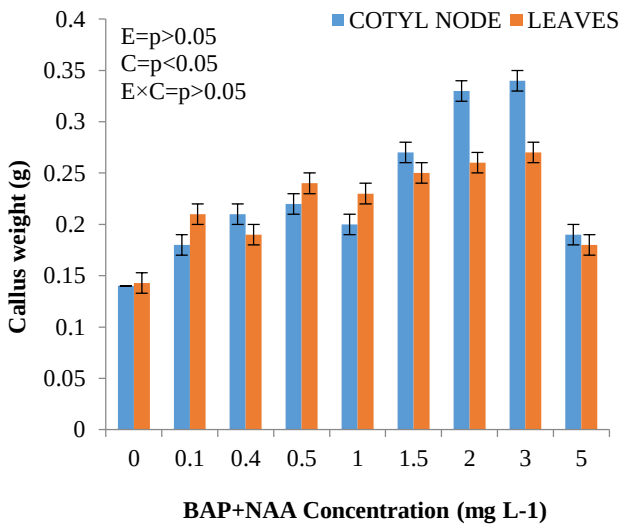

Fig. 1b. Callus induction in Glycine max using various combinations and concentrations of BAP and NAA.

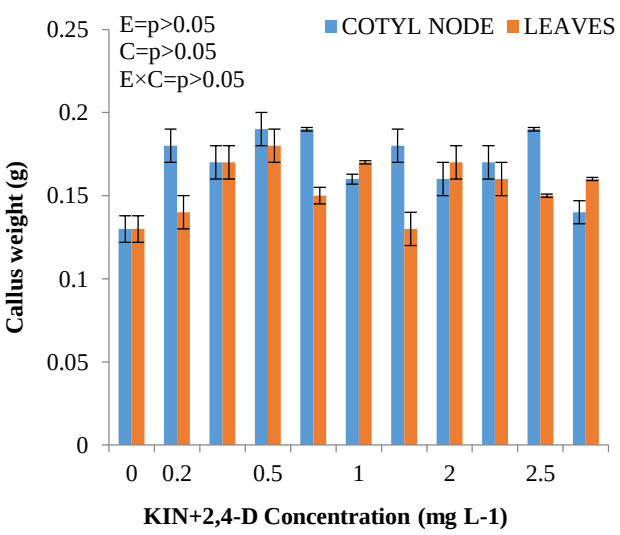

Fig. 1c. Callus induction in *Glycine max* using various combinations and concentrations of KIN and 2, 4-D.

Table 1. The effect of different combinations of cytokinin and auxins on regeneration of shoots from callus, produced from cotyledonary node of *Glycine max* after 25 days.

Hormone	Conc. (mg L^{-1})	Response %	Mean No. of shoots ± SD	Mean length of shoots (cm) ± SD
Control	0.0	0.0	0.0	0.0
KIN	1.0	47	1.20 ± 0.007	1.50 ± 0.01
	2.0	35	1.03 ± 0.02	1.66 ± 0.02
	3.0	33	0.80 ± 0.01	1.70 ± 0.01
BAP	0.5	52	1.07 ± 0.03	1.20 ± 0.01
	1.0	44	0.94 ± 0.05	1.12 ± 0.02
	1.5	56	1.03 ± 0.06	1.22 ± 0.005
	2.0	57	1.17 ± 0.01	1.22 ± 0.06
	2.5	30	1.09 ± 0.02	1.33 ± 0.03
	3.0	41	0.93 ± 0.06	1.06 ± 0.005
	3.5	47	1.23 ± 0.08	1.36 ± 0.003
	4.0	46	1.04 ± 0.09	1.25 ± 0.01
	4.5	48	1.19 ± 0.08	1.29 ± 0.02
	5.0	45	1.15 ± 0.005	1.23 ± 0.01
BAP + KIN	1.0 + 1.0	62	1.33 ± 0.08	1.80 ± 0.03
	2.0 + 1.0	88	3.00 ± 0.09	4.22 ± 0.08
	3.0 + 1.0	69	2.66 ± 0.04	2.33 ± 0.05
	4.0 + 1.0	58	1.00 ± 0.06	1.16 ± 0.03
BAP + NAA	0.4 + 0.4	44	1.6 ± 0.06	1.5 ± 0.03
	1.0 + 1.0	42	1.2 ± 0.03	1.6 ± 0.01
	1.5 + 1.5	39	1.8 ± 0.02	1.9 ± 0.03
	2.0 + 2.0	30	1.3 ± 0.01	1.0 ± 0.02

Shoot formation: The cotyledonary nodal callus was transferred to regeneration medium for shoot induction. MS with combination of BAP+KIN (2.0+1.0 mg L^{-1}) showed significant shoot induction and maximum shoot elongation (Table. 1). The maximum number and length of shoots were recorded 3.00±0.09 and 4.22±0.08 cm receptively. KIN alone showed maximum number of shoots on 1.0 mg L^{-1} and maximum length of shoots produced at 3.0 mg L^{-1}. BAP showed maximum numbers and length of shoot at 3.5 mg L^{-1} while combinations of BAP+NAA induced both maximum number and length of shoots at 1.5+1.5 mg L^{-1}.

Root formation: MS media supplemented with IBA (0.5 mg L^{-1}) concentration showed highest roots formation.

Discussion

On the response basis, MS medium used for induction of callus, shoot and root formation and multiplication with different concentration of auxins and cytokinins. The best response showed from cotyledonary node with abundant callus (Fig. 1a, 1b). The cotyledonary node explants were used to produce calli and shoots on the MS medium. The cotyledonary node explants acted as potential candidate for transformation and regeneration studies in *Glycin max* (Liu *et al.,* 2010) and also in other plants (Zhang *et al.,* 2011). Plant growth regulators have been used for cell division and differentiation. Regarding callus initiation, explants were provided different concentration of phytohormones such as; 2, 4-D and combinations of BAP, KIN and cytokinins. The callus was friable and yellowish in colour and produced on all plant hormone after 12-14 days of inoculation but the abundant callus was formed on 2, 4-D (4.0 mg L^{-1}) from cotyledonary node explants. It is reported that the 2, 4-D is a commonly used auxin for induction of callus and somatic embryogenesis and is a better option as compared to other hormones (Mariashibu *et al.,* 2013; Loganathan *et al.,* 2010; Naz *et al.,* 2018). Different combinations of auxins and cytokinins were also used for callus induction but there was no significant formation of callus produced as compared to 2, 4-D alone. The shoots were induced from cytokinins and combination of auxins and cytokinins. In this study, high frequency of emerging shoots from cotyledonary node was found on combination of BAP plus KIN (2.0+1.0 mg L^{-1}) with highest shoots number (3.00±0.09) and length (4.22±0.08cm) (Table 1). Hence, this combination (BAP + KIN) served as an excellent combination for shoot induction, as reported in sugarcane (Ali *et al.,* 2008) *Physalis minima* L. (Mungole *et al.,* 2011) Strawberry (Sakila *et al.,* 2007). The shoots were transferred on rooting media and the best roots were formed at IBA (0.5 mg L^{-1}). *Glycine max* produced maximum roots at IBA concentration (14.77 µ M) (Radhakrishnan & Ranjithakumari, 2007) that would be helpful for farmers of this species. Consequently the country would be benefitted.

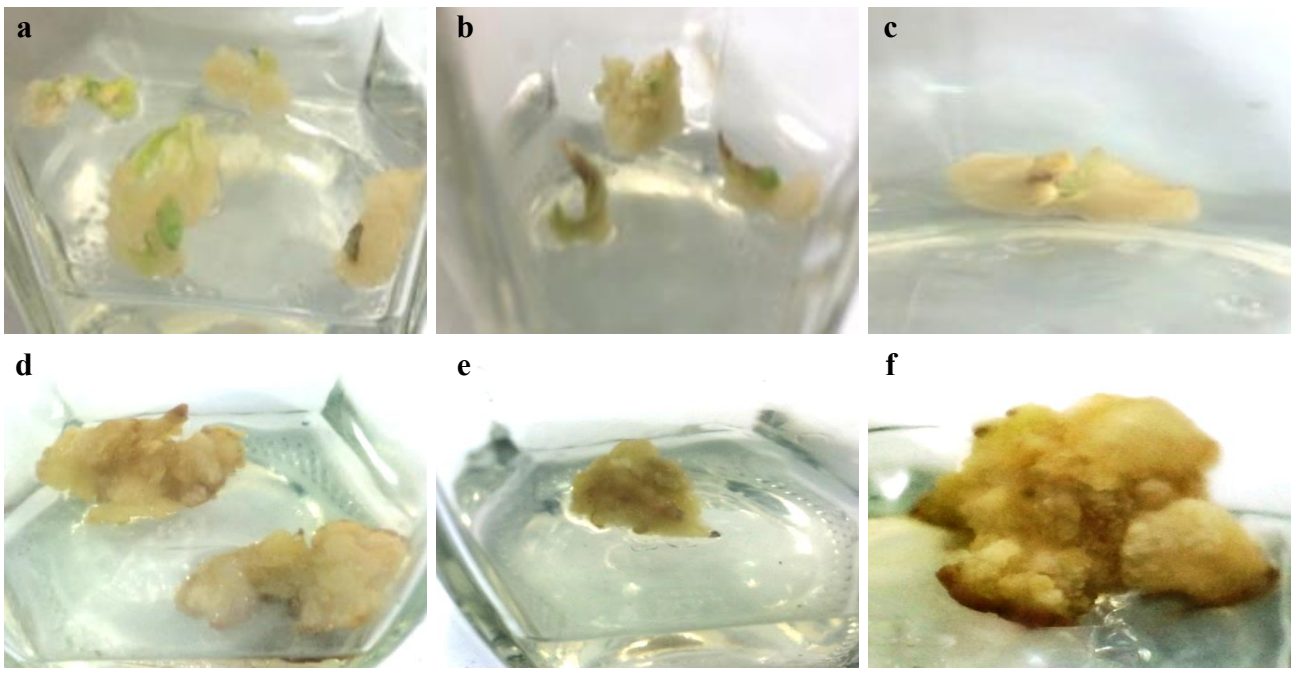

Fig. 1d. Initiation of callus from cotyledonary node tissues of *Glycine max* on different concentrations of 2, 4-D on MS medium for 25 days. (a,b) inition of callus after 10-12 days from different explants (c); cotyledonay node callus after 14 days on1.5 mg L^{-1} 2, 4-D; (d) 2.5 mg L^{-1} 2, 4-D; (e) 3.5 mg L^{-1} 2, 4-D; (f) 4.0 mg L^{-1} 2, 4-D.

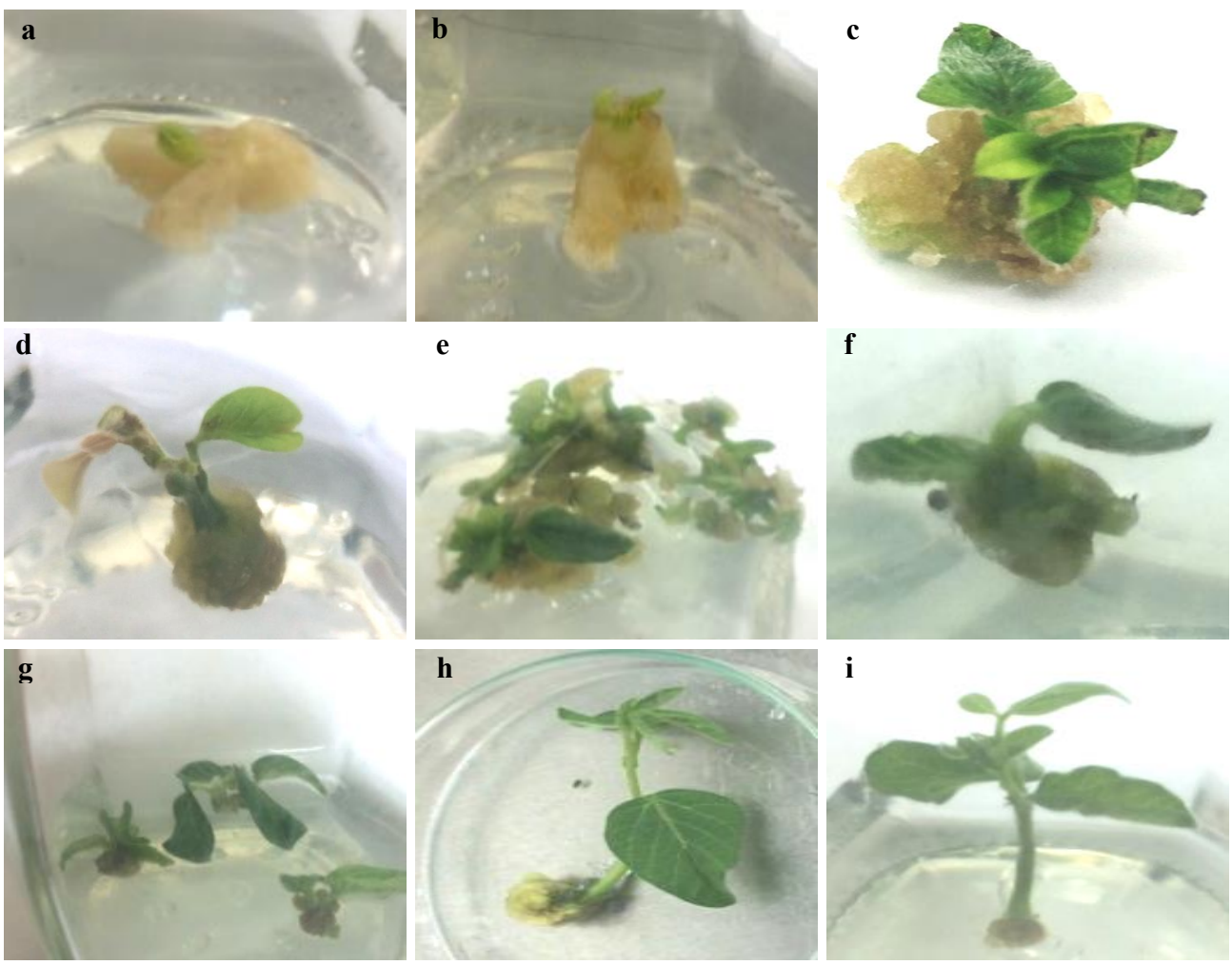

Fig. 1e. Initiation of shoots from callus of *Glycine max* on BAP+KIN (2.0+1.0 mg L^{-1}) on MS medium after 25 days. (a,b) Initiaon of shoots from callus after 7 days (c,d,e,f,g) ; Regenerated shoots formed cotyledonay node callus after 14-18 days on BAP+KIN; (h) Shoots on BAP+KIN (2.0+1.0 mg L^{-1}) after 22 days.; (i) Regenerated plants after 25 days.

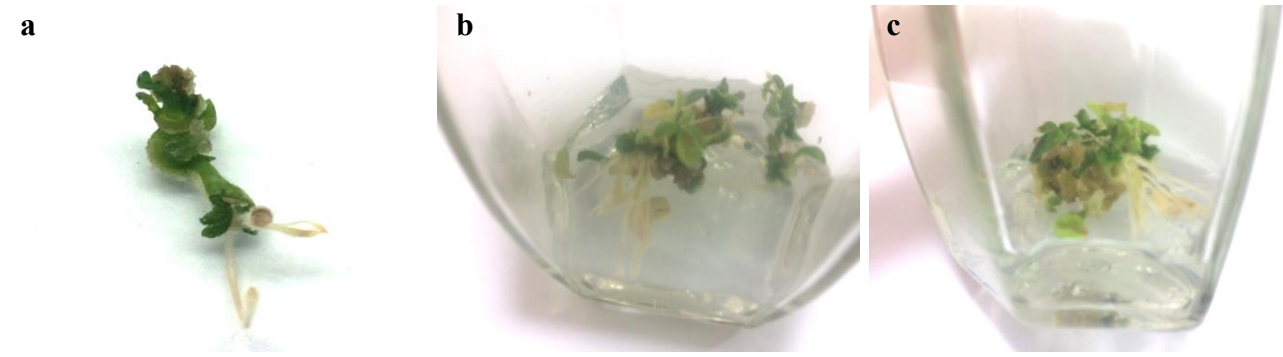

Fig. 1f. Regenerated roots on IBA 0.5 mg L^{-1} (a, b, c) Regenerated plants after 25 days.

Conclusion

In present study, we established a useful micro propagation protocol for *In vitro* regeneration of *Glycine max* from cotyledonary node.The utility of cotyledonary nodes as explant for highest percentage of induction of callus on 2, 4-D (4.0 mg $L^{-1)}$ and the maximum shoot induction established on combination of BAP (2.0 mg L^{-1}) plus KIN (1.0 mg L^{-1}) was the most efficient for *Glycine max* regeneration and seedling establishment. Overall, efficiency of this system play important function in the development of genetically modified crop plants using Agrobacterium-mediated T-DNA transfer.

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