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In-vitro Culturability of Soybean Cultivars

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Abstract

In-vitro regeneration of soybean is a prerequisite for successful genetic transformation. This study aimed to investigate the culturability of three soybean cultivars for *in-vitro* regeneration, focusing on genotypes, media, explant types, and plant growth regulators. Shoot tips, cotyledons, and hypocotyl segments were excised from 7-day-old *in-vitro* seedlings as explants for subsequent *in-vitro* regeneration. All cultivars exhibited good callus formation from all types of explants, with shoot tips and cotyledons being more suitable. Calluses were induced on Gamborg B5 or Murashige and Skoog (MS) media using various concentrations of 6-benzyladenine (BA) and thidiazuron (TDZ) or 2,4-dichlorophenoxyacetic acid (2,4D). The combination of BA and TDZ or BA and 2,4D exerted an inhibitory effect on shoot and root induction. Media supplemented with BA produced calluses at a rate of 100%. Appropriate BA concentrations for shoot induction for ĐT35 and VNUAĐ2 were 1.0-2.0 mg L^{-1}, and 2.0-3.0 mg L^{-1}, respectively. MS + 2 mg L^{-1} BA for shoot tip explants and MS + 3.0 mg L^{-1} BA for cotyledon explants were appropriate to induce calluses and shoots. As BA concentrations increased, the root induction rate gradually decreased considerably. For both ĐT35 and VNUAĐ2, MS + 2.0 mg L^{-1} BA + 0.5 or 1.0 mg L^{-1} αNAA could be used to multiply calluses. Although shoots induced from calluses of ĐT35 were observed on MS + 1.5 or 2.0 mg L^{-1} BA + 1 mg L^{-1} αNAA, no shoots were observed for VNUAĐ2.

Keywords

Callus, cotyledon, *in-vitro,* growth regulators, shoot tip

Introduction

Soybean (*Glycine max* (L.) Merr.), a member of Fabaceae family, is one of the world's most important vegetable protein and oil sources. Seed yield, nutritional quality, and resistance to various insects and diseases are important objectives in soybean amelioration (Jiang *et al*., 2023). However, the genetic base of soybean cultivars

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is relatively narrow (Hiromoto & Vello, 1986), with most current varieties tracing back to a small number of parents. Thus, genetic gains or the rate of improvement via traditional breeding are generally low. With advances in molecular genetics, powerful tools can be used to expand the genetic base and develop materials with desirable traits for both qualitative and quantitative selection (Petereit *et al*., 2022). For soybean, genetic transformation represents an invaluable tool for molecular breeding programs, allowing the production of novel and genetically diverse genotypes for selection, which are otherwise difficult through conventional breeding. However, soybean is a crop recalcitrant to genetic manipulation and *in-vitro* regeneration (Kantayos & Bae, 2019; Mangena, 2020). Hence, an efficient and stable transformation procedure for soybean cannot yet be considered routine because it depends on the ability to combine efficient transformation and reproducible *in-vitro* regeneration techniques. Genotype specificity, culture conditions, and types of explants are important factors that require optimization before an efficient system of regeneration can be developed (Bidabadi *et al.,* 2020). The establishment of an *in-vitro* regeneration procedure for recalcitrant legumes like soybean is still a challenge (Singh *et al.*, 2020). Therefore, an efficient and reproducible plant regeneration protocol is essential for the successful recovery of genetic transformants from friable calluses in soybean (Mangena, 2020).

Previous studies have suggested that different genotypes behave differently when treated in-vitro and, thus, the medium and plant growth regulators (PGRs) to culture explants from different commercial cultivars need to be investigated (Singh *et al.*, 2020). Murashige and Skoog (MS) and Gamborg B5 vitamin (GB5) media are commonly used in *in-vitro* culture (Vu Thi Thu Hien & Vu Thi Thuy Hang, 2021). Common PGRs used for soybean regeneration are cytokinins, such as 6-benzyladenine (BA), 6-benzylaminopurine (BAP), and thidiazuron (TDZ), or auxins, such as 1-Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4D) (Singh *et al.*, 2020). Cytokinin and auxin regulators stimulate shoot and root development, respectively (Sosnowski *et al.*, 2023). For example, Bonacin *et al.* (2020) used cotyledons to study the embryogenic capability of five soybean cultivars using 8, 10, and 12 mg L^{-1} NAA. Raza *et al.* (2017) used three different explants (half-split hypocotyls, complete hypocotyls, and cotyledonary nodes) of nine soybean cultivars to regenerate shoots. Variations in shoot induction and regeneration rates were observed with a range of 50-100% and 2.6-10 shoots/explant, respectively, on GB5 or MS + 1.67 mg L^{-1} BAP media (Raza *et al.*, 2017). Various studies have used almost all parts of the plant as explants for regeneration, including cotyledonary nodes, stem internodes, epicotyl sections, tissues from primary leaves, plumules, hypocotyls, immature cotyledons, embryos, and roots (see Soto *et al.*, 2013). However, previous research has shown that *in-vitro* regeneration and successful genetic transformation are frequently based on explants/plant organs related to cotyledons (Raza *et al.*, 2017; Singh *et al.*, 2020). Regardless of the transformation method, regeneration *in-vitro* via organogenesis or somatic embryogenesis is a required step. Since different genotypes or cultivars and explants respond differently to PGRs, it is necessary to develop an appropriate *in-vitro* protocol for each genotype before conducting transformation. This research aimed to assess the culturability of improved soybean cultivars and to develop efficient morphogenesis/organogenesis (callus, micro-shoot, and root induction) conditions and regeneration systems using shoot tips, hypocotyl segments, and cotyledons for future genetic manipulation and improvement of soybean.

Materials and Methods

Plant materials

Seeds of three soybean cultivars (DT2008, ĐT35, and VNUAĐ2) were used in this study. DT2008, ĐT35, and VNUAĐ2 were bred by Agricultural Genetics Institute, Food Crops Research Institute, and Vietnam National University of Agriculture, respectively. DT2008 was released in 2016, ĐT35 was released in 2021, and VNUAĐ2 obtained certification of plant variety protection in 2021.

Two hundred healthy seeds (uniform in size and color, disease-free, and without cracks) selected from each of three soybean cultivars were washed thoroughly three times with distilled water containing 0.6% Tween 80 and then surface sterilized with 0.5% Presept for 15min, followed by three rinses with sterile distilled water under a laminar air flow cabinet, and dried with sterile filter paper. Seeds were then peeled, further sterilized with 0.5% Presept for 10min, washed three times with sterile distilled water, and dried with sterile filter paper before being placed in jars containing 40 ml of ½ MS (Murashige & Skoog, 1962). Jars with 5 seeds/jar were kept under a 16/8 h light/day cycle at 25°C with a light intensity of 2000 lux. Healthy seedlings after a 7-day culture period were used for harvesting explants, shoot tips, cotyledons, and hypocotyl segments for the next experiment **(Figure 1).**

Experiment 1. Effect of media, plant growth regulators (PGRs), and explant types on callus, shoot, and root induction

Two modified basal media (pH 5.8) were used: GB5 (Gamborg *et al*., 1968) + 30 g L^{-1}sucrose + 7 g L^{-1} agar (hereafter referred to as GB5) and MS + vitamins + 30 g L^{-1} sucrose + 7 g L^{-1} agar (hereafter referred to as MS). GB5 was supplemented with 6-benzyladenine (BA) and thidiazuron (TDZ) while MS was supplemented with BA and 2,4-dichlorophenoxyacetic acid (2,4D) (**Table 1**).

Shoot tips, cotyledons, and hypocotyl segments were taken from seedlings of DT2008, ĐT35, and VNUAĐ2 cultured on ½ MS medium for 7 days (**Figure 1**). The total number of explants varied depending on the number of available healthy seedlings obtained from the preparation of *in-vitro* seedlings. Two to three explants were placed in each jar and kept under a 16/8 h light/dark cycle, 2000 lux light intensity, 25°C temperature, and 60-70% relative humidity. The rates of explants producing calluses, shoots, and roots were recorded weekly during the 6 weeks of culture.

Experiment 2. Effect of BA and explant types on organogenesis of ĐT35 and VNUAĐ2

Based on results of experiment 1, two types of explants, shoot tips, and cotyledons, were prepared from the in-vitro seedlings of ĐT35 and VNUAĐ2. Four cotyledons were placed in each of

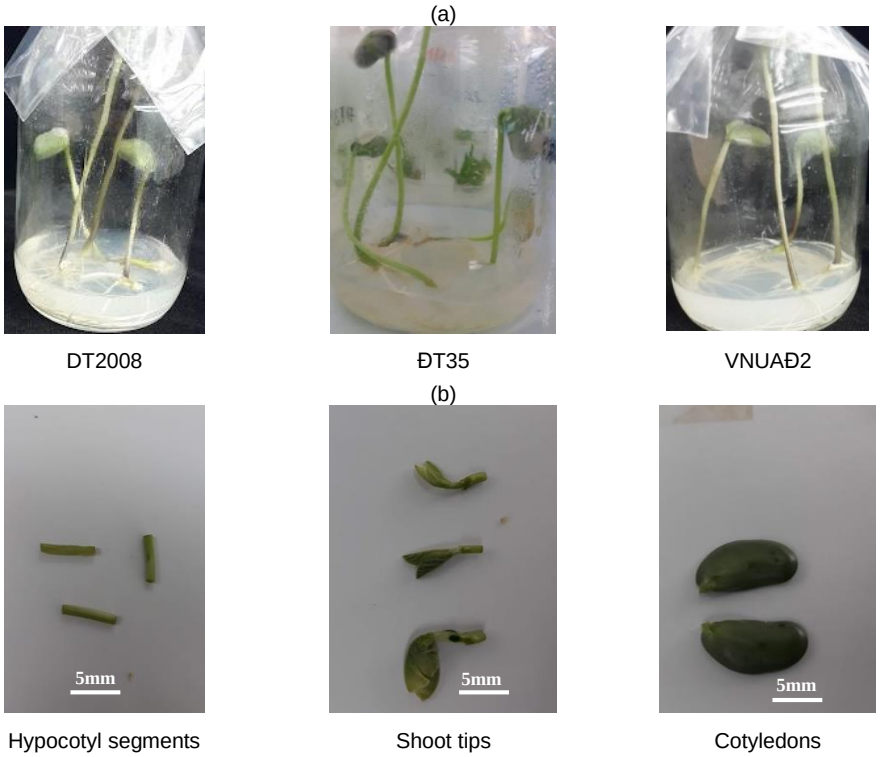


Figure 1. (a) *In-vitro* seedlings of three soybean cultivars 7 days after culture; (b) three types of explants

Table 1. GB5 and MS media with different plant growth regulator combinations

Media	Plant growth regulators	
(a) GB5 with BA and TDZ	BA (mg L^{-1})	TDZ (mg L^{-1})
GB5 + 30 g L^{-1} sucrose + 7 g L^{-1}agar	0	0
	1.0	0
	1.5	0
	2.0	0
	0	1.0
(b) MS with BA and 2,4D	BA (mg L^{-1})	2,4D (mg L^{-1})
MS + vitamin + 30 g L^{-1} sucrose + 7 g L^{-1}agar	0	0
	1.0	0
	1.0	3.0
	1.5	0
	2.0	0
	2.5	0

10 jars and two shoot tip explants (0.7-1cm) were were placed in each of 15 jars containing corresponding BA concentration (MS + 30 g L^{-1} sucrose + 7 g L^{-1} agar supplemented with BA concentrations of 0, 1, 2, 3, or 4 mg L^{-1}). Jars were kept in conditions similar to experiment 1 and checked weekly during 6 weeks of culture.

Experiment 3. Effect of BA and α-naphthaleneacetic acid (α-NAA) on morphogenesis of calluses of ĐT35 and VNUAĐ2

MS medium + 30 g L^{-1} sucrose + 7 g L^{-1} agar containing different concentrations of BA and α-NAA (**Table 2**) were used to evaluate effects of PGRs on shoot and root induction from calluses. Large-sized, uniform, and blue-colored calluses of ĐT35 and VNUAĐ2 derived from the previous experiment (Figure 2a) were aseptically cut into small blocks 5.0mm in size (**Figure 2b**). Three callus blocks were then placed in each of 10 jars containing the corresponding PGR combination. Jars were kept in conditions similar to the previous experiment, checked weekly, and assessed after 6 weeks of culture.

Recorded data

After 6 weeks of culture, inductions of calluses, shoots, and roots were recorded. The numbers of explants induced by calluses, shoots, and roots were counted. Characteristics of induced calluses, shoots, and roots, namely size, color, and structure, were recorded. Calluses were described as small, medium, and large if the sizes were ≤ 2.5mm, 2.5-5.0mm, and > 5.0mm, respectively. Calluses could have different colors (e.g. green, brown, creamy green, and white margins) and a compact or soft structure. Induced shoots could be small or in clusters. Induced roots were small, white, or well-developed with lengths of 3-5cm.

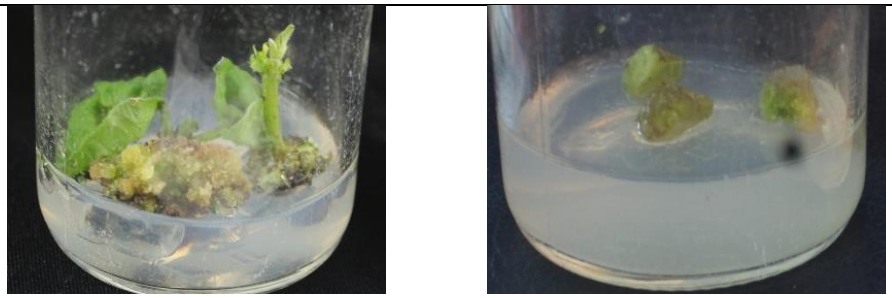
Data analysis

Data gathered was compiled using Excel 10 and statistically analyzed via ANOVA using Infostat. Least significant difference (LSD) (P <0.05) was calculated and used for means comparison. Induction rates (%) of calluses, shoots, and roots were calculated as ratios of the number of explants forming calluses, shoots, and roots over the total number of explants.

Results and Discussion

Effect of culture media and explant type on organogenesis of DT2008, ĐT35, and VNUAĐ2 (Experiment 1)

Explants of shoot tips, cotyledons, and hypocotyl segments were used to examine effects of GB5 and MS media with different



(a) 6 week-old calluses for subculture (b) subculture of callus blocks

Figure 2. Six week cultured calluses and callus blocks subcultured on regeneration medium

Table 2. Combinations of BA and αNAA on MS

BA (mg L^{-1})	α-NAA (mg L^{-1})
0	0.5
1.5	0.5
2.0	0.5
2.5	0.5
0	1.0
1.5	1.0
2.0	1.0
2.5	1.0

concentrations of PGRs (**Table 3; Figure 3**). The survival rate after 6 weeks of culture was 100%. Explants on both types of media resulted in a similar callus induction rate (~95%), but the average root and shoot formation rates were slightly higher in GB5 than in MS, 29.2% and 11.2%, respectively. The highest percentage of shoot (28.6%) and root (92.9%) induction were recorded in GB5 without PGR.

MS resulted in high callus formation (95.4%), and the average shoot and root induction rates were 10.3% and 20.7%, respectively. MS + 2.5 mg L^{-1} BA had the highest shoot induction rate (23.1%), while MS + 2.0 mg L^{-1} BA had no shoot or root formation. MS without PGRs resulted in a significanlty high root induction rate (85.7%) **(Table 3)**.

TDZ and 2,4D did not influence callus induction but completely inhibited shoot and root formation. In contrast, Franklin *et al.* (2004) reported that the presence of BAP and TDZ in MS exerted a synergistic effect, and the regeneration efficiency was higher than for cytokinin alone. Arvinth *et al.* (2008) showed that MS + 2 mg L^{-1} 2,4D was the best for callus production, and MS + 2 mg L^{-1} BAP + 0.1 mg L^{-1} NAA was suitable for shoot proliferation. Overall, GB5 + 1.5-2.0 mg L^{-1} BA and MS + 1.0 or 2.5 mg L^{-1} BA were appropriate to induce calluses, shoots, and roots depending on specific cases.

The morphogenesis of different explant types was significantly different, but not among DT2008, ĐT35, and VNUAĐ2 **(Table 4).** For callus induction, cotyledons produced the highest callus rate in all three cultivars (100%). Shoot tips produced high callus rates in DT2008 and ĐT35, and hypocotyl segments produced a high callus rate in VNUAĐ2. For shoot induction, shoot tips produced the highest shoot rates, with 69.2% in DT2008, 50.0% in ĐT35, and 37.5% in VNUAĐ2. For root induction, cotyledon explants gave the highest average root induction (40.2%), with the highest being in ĐT35 (58.3%).

Overall, media supplemented with BA resulted in calluses with a larger size, blue color, and strong and solid structure, and short shoots, but poor root development. In contrast, media

Table 3. Influence of media and plant growth regulators on morphogenesis of three soybean cultivars 6 weeks after culture

Medium		No. of explants observed	Callus rate (%)	Shoot rate (%)	Root rate (%)
GB5 with BA and TDZ					
BA (mg L^{-1})	TDZ (mg L^{-1})				
0	0	14	92.9^{ab}	28.6^{c}	92.9^{d}
1.0	0	19	85.0^{a}	5.0^{ab}	15.0^{b}
1.5	0	19	100^{b}	15.8^{b}	15.8^{bd}
2.0	0	16	100^{b}	11.7^{ab}	41.2^{c}
0	1	20	100^{b}	0.0^{a}	0.0^{a}
MS medium with BA and 2,4D					
BA (mg L^{-1})	2,4D (mg L^{-1})				
0	0	14	85.7^{a}	14.3^{b}	85.7^{d}
1.0	0	15	100^{b}	20.0^{bc}	20.0^{bd}
1.0	3.0	16	100^{b}	0.0^{a}	0.0^{a}
1.5	0	16	100^{b}	6.3^{ab}	12.5^{ab}
2.0	0	14	85.7^{a}	0.0^{a}	0.0^{a}
2.5	0	13	100^{b}	23.1^{bc}	7.7^{ab}
Grand total		176	95.5	10.8	25.0
GB5		89	95.5	11.2	29.2
MS		87	95.4	10.3	20.7
$LSD_{0.05\ media}$			8.15	12.07	13.77

Note: Results were the average of three soybean cultivars and of explants for each corresponding medium. Values with the same superscript small letter in the same column are not significantly different at 5% probability level.

without BA resulted in small and brown calluses, long shoots, and good root development. Treatments with BA from 1.0-2.5 mg L^{-1} were not different statistically. Of the two media, MS appeared more effective for callus morphology than GB5. Calluses in MS medium were larger and green, while calluses in GB5 medium were small with a black margin **(Figure 4)**. Thus, MS was suitable for *in-vitro* soybean culture. Additionally, shoot tips and cotyledon explants resulted in relatively high morphogenesis rates compared to hypocotyl segments in ĐT35 and VNUAĐ2 **(Table 4)**.

Since DT2008 has a longer cultivation history and has been studied for *in-vitro* regeneration (Dang Trong Luong *et al.*, 2013), this study focused on developing *in-vitro* regeneration for the two newly released cultivars, ĐT35 and VNUAĐ2. Thus, MS medium, shoot tips, and cotyledons of ĐT35 and VNUAĐ2 were selected for subsequent experiments with BA.

Effect of BA and explant types on organogenesis of ĐT35 and VNUAĐ2 (Experiment 2)

For ĐT35, 100% of explants survived and formed calluses, shoots, and roots **(Table 5)**. All the media supplemented with BA produced calluses using both shoot tips and cotyledons as explants, which was significantly different from the control (50% for shoot tips and 22.9% for cotyledons).

Shoot tip explants produced the highest average shoot rate at BA concentrations of 0, 1, and 2 mg L^{-1} (100%) and the lowest at 4 mg L^{-1} (38.9%). Shoot tip explants produced the highest root rate of 80% without BA, which was significantly different from the BA treatments.

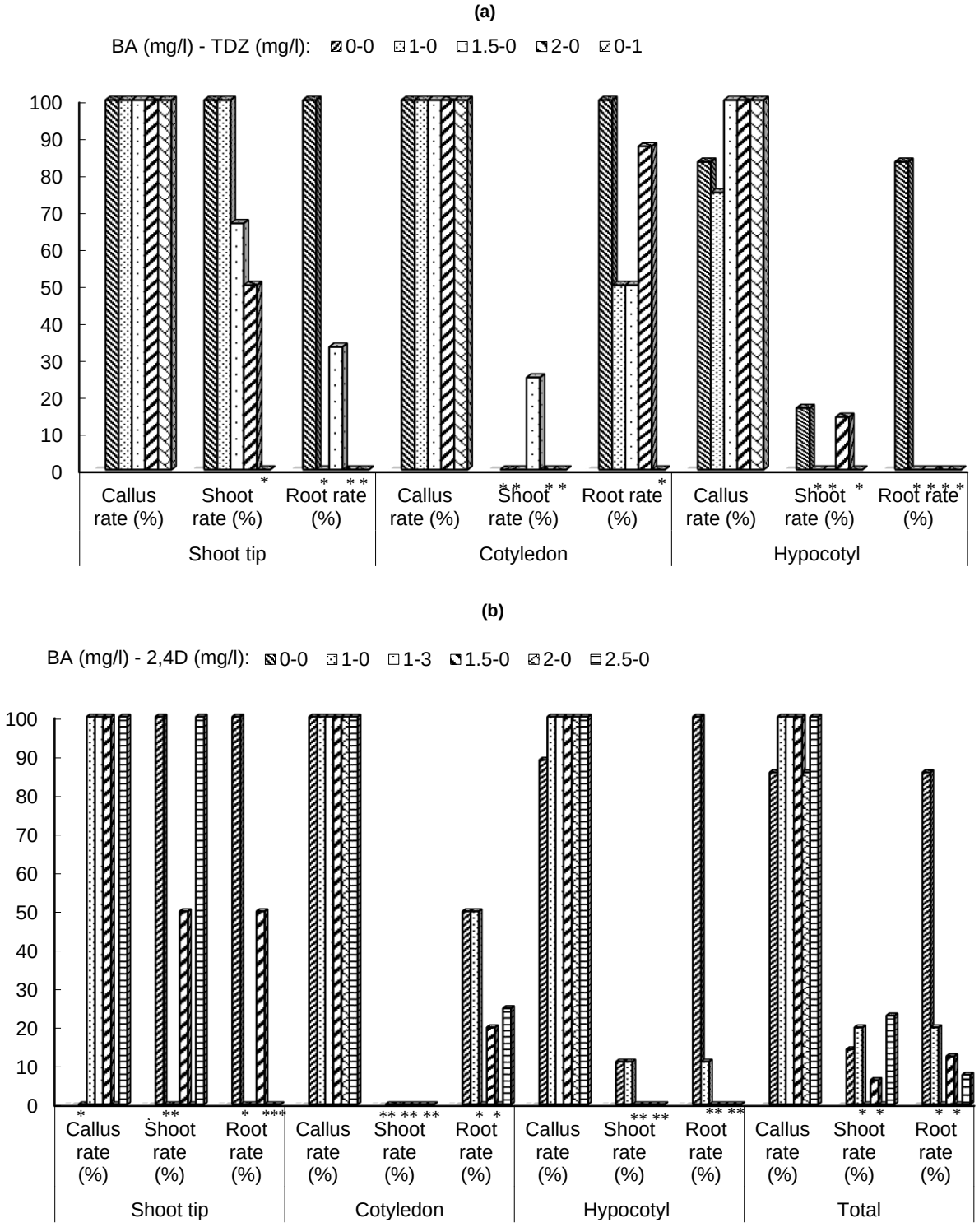


Figure 3. Influence of media, plant growth regulators, and explants (shoot tips, cotyledons, and hypocotyls) on morphogenesis of three soybean cultivars 6 weeks after culture: (a) GB5 + BA (0 - 2 mg L^{-1}) + TDZ (0 - 1 mg L^{-1}); (b) MS + BA (0 - 2.5 mg L^{-1}) + 2,4D (0 - 3 mg L^{-1}). Results were the average of three soybean cultivars for each explant type for each corresponding medium. (*) indicates 0% induction rate.

Thus, BA concentrations up to 2 mg L^{-1} improved the callus induction rate, but when the concentration exceeded 2 mg L^{-1}, shoot induction decreased accordingly.

Table 4. Influence of cultivars and explant types on organogenesis of three soybean cultivars 6 weeks after culture

Cultivar	Explant types	No. explants observed	Callus rate (%)	Shoot rate (%)	Root rate (%)
DT2008	Shoot tip	13	100	69.2	23.1
	Cotyledon	32	100	0.0	37.5
	Hypocotyl	57	96.5	7.0	17.5
	Total	102	98.0	12.7	24.5
ĐT35	Shoot tip	4	100	50.0	25.0
	Cotyledon	12	100	8.3	58.3
	Hypocotyl	15	80.0	0.0	13.3
	Total	31	90.3	9.7	32.3
VNUAĐ2	Shoot tip	8	62.5	37.5	25.0
	Cotyledon	14	100	0.0	28.6
	Hypocotyl	21	100	0.0	14.3
	Total	43	93.0	7.0	20.9
Grand total		176	95.5	10.8	25.0
Shoot tip		25	88.0	52.9	23.5
Cotyledon		58	100	2.0	40.2
Hypocotyl		93	94.6	4.0	16.5
$LSD_{0.05\ Cultivar}$			8.64	12.80	14.61
$LSD_{0.05\ Explants}$			8.15	12.07	13.77
$LSD_{0.05\ Cultivar \times Explant}$			15.24	22.58	25.77

Note: Values were the average of different media corresponding to three soybean cultivars and explants.

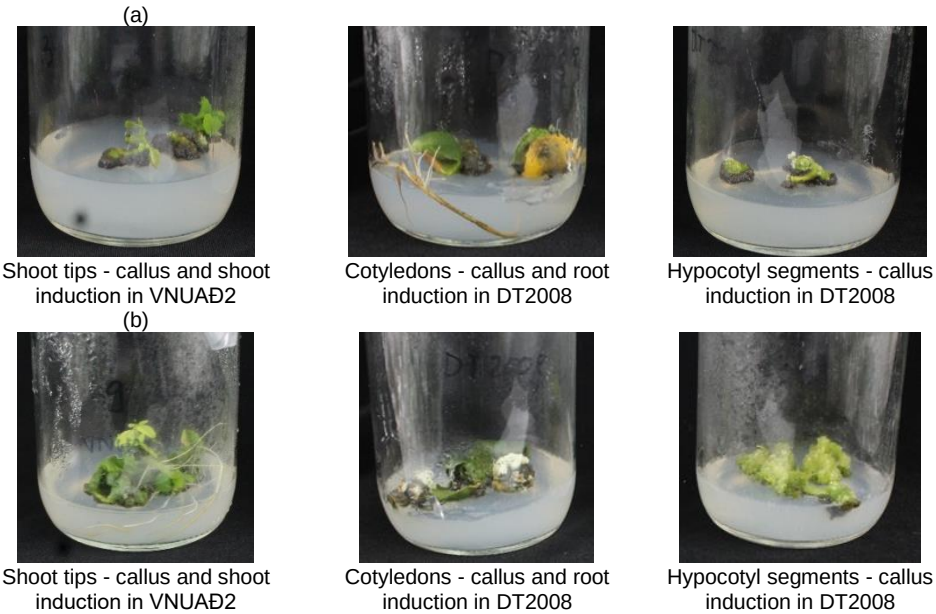


Figure 4. Morphogenesis of different explant types cultured on (a) GB5 + 1.5 mg L^{-1} BA; and (b) MS + 1.5 mg L^{-1} BA after 6 weeks of culture in VNUAĐ2 and DT2008

Cotyledon explants produced the highest average shoot induction rate (100%) without BA, which was non-significantly different from 1 mg L^{-1} BA. Similarly, the shoot induction rate

decreased considerably as the BA concentration increased (53.6% at 4 mg L^{-1}). When the BA concentration increased, the root induction rate decreased and there was no root induction at 3 mg L^{-1}.

For VNUAĐ2, media supplemented with BA produced calluses of 100% using both shoot tips and cotyledons as explants, which was significantly different from the control (38.5% for shoot tip and 75.0% for cotyledons). Shoot

Table 5. Influence of BA and explant types on organogenesis of ĐT35 and VNUAĐ2

Cultivar/explant	BA (mg L^{-1})	Total explants observed	Rate of callus induction (%)	Rate of shoot induction (%)	Rate of root induction (%)
Shoot tip	0	20	50.0^{b}	100^{ef}	80.0^{b}
	1	22	100^{d}	100^{ef}	13.6^{a}
	2	20	100^{d}	100^{ef}	0.0^{a}
	3	26	100^{d}	80.8^{c}	3.8^{a}
	4	18	100^{d}	38.9^{a}	0.0^{a}
Cotyledon	0	32	22.9^{a}	100^{ef}	87.5^{b}
	1	28	100^{d}	92.9^{d-f}	78.6^{b}
	2	40	100^{d}	70.0^{bc}	5.0^{a}
	3	36	100^{d}	63.9^{bc}	0.0^{a}
	4	28	100^{d}	53.6^{ab}	3.6^{a}
Shoot tip	0	26	38.5^{ab}	100^{ef}	84.6^{b}
	1	24	100^{d}	58.3^{a-c}	8.3^{a}
	2	20	100^{d}	100^{ef}	20.0^{a}
	3	26	100^{d}	100^{ef}	0.0^{a}
	4	22	100^{d}	72.7^{bc}	0.0^{a}
Cotyledon	0	32	75.0^{c}	100^{ef}	100^{b}
	1	28	100^{d}	81.3^{c-f}	100^{b}
	2	24	100^{d}	80.0^{b-f}	16.7^{a}
	3	28	100^{d}	92.9^{d-f}	3.6^{a}
	4	20	100^{d}	100^{ef}	20.0^{a}
Means for cultivar	ĐT35	270	87.3	80	28.5
	VNUAĐ2	250	91.3	88.5	33.7
Means for explants	Shoot tip	224	88.8	85.1	21.0
	Cotyledon	296	89.8	83.4	41.1
Means for BA (mg L^{-1})	0	110	46.6^{A}	100^{C}	91.2^{C}
	1	102	100^{B}	83.1^{B}	50.1^{B}
	2	104	100^{B}	87.5^{B}	6.25^{A}
	3	116	100^{B}	84.4^{B}	1.85^{A}
	4	88	100^{B}	66.3^{A}	5.89^{A}
$LSD_{0.05\ Explant}$			5.20	7.94	6.45
$LSD_{0.05\ BA}$			8.09	12.35	10.02
$LSD_{0.05\ Cultivar}$			5.10	7.77	6.31
$LSD_{0.05\ Cultivar \times BA \times Explant}$			16.74	25.5	20.74

Note: Values with the same superscript small or capital letters in the same column are not significantly different at 5% probability level.

tip explants produced the highest shoot rate (100%) at 0, 2, and 3 mg L^{-1} BA and the lowest (58.3%) at 1 mg L^{-1} BA. Shoot tip explants produced the highest root rate of 84.6% in media without BA, which was significantly different from BA treatments. This indicated that when BA was within 0-3 mg L^{-1}, shoot induction increased but then decreased at higher concentrations (> 3 mg L^{-1}). Cotyledon explants produced the highest shoot rate (100%) without BA, while root induction decreased as BA exceeded 1 mg L^{-1}.

Of the two soybean cultivars, VNUAĐ2 exhibited better culturability in terms of callus, shoot, and root induction. VNUAĐ2 showed an average callus induction rate of 91.3%, higher than ĐT35 by 4% but not significantly different. Shoot induction in VNUAĐ2 reached 88.5%, 8.5% higher than ĐT35, while root induction in both cultivars was similar.

Morphogenesis rates of the two cultured explants, shoot tips and cotyledons, were different. Cotyledon explants produced slightly higher callus rates and lower shoot rates than those of shoot tip explants. However, cotyledon explants were superior to shoot tips in terms of root induction (41.1% vs 21%).

BA stimulated callus and shoot induction but inhibited root induction in soybean. Media supplemented with BA produced calluses of 100%, significantly higher than treatments without BA. High BA concentration inhibited shoot formation. The appropriate BA concentration for shoot induction in ĐT35 was 1.0-2.0 mg L^{-1}, while that for VNUAĐ2 was in the range of 2.0-3.0 mg L^{-1}. Additionally, in the medium without BA, induced shoots were long, thin, and did not have many branches. In the media supplemented with BA, shoots were thick and short with shoot clusters. BA concentrations of 2 mg L^{-1} for shoot tips and 3 mg $L^{-1}$for cotyledons were suitable for shoot formation. At higher BA concentrations, induced shoots were thin and short, or did not even form shoots.

In the medium without BA, roots were long and branched, covering the bottom of the jar, but in the media supplemented with BA, the root formation rate was poor (**Figure 4**). As the BA concentration increased, the root induction rate gradually decreased and reached 0% at a concentration of 3 mg L^{-1}.

Additionally, calluses induced with MS + 2 mg L^{-1} BA for shoot tips and MS + 3 mg L^{-1} BA for cotyledon were the best with a large size and green color **(Figure 5)**. Thus, these calluses were collected and used for the subsequent experiment.

BA as a cytokinin plays various principal functions in plant development, morphogenesis, and plant tissue culture, including the *in-vitro* regeneration of soybeans (Mangena, 2020). Zia *et al*. (2010) and Raza *et al.* (2017) also concluded that cotyledonary node explants were more efficient for *in-vitro* plant regeneration and among variants of cytokinins, BA was better compared to zeatin riboside and kinetin for regeneration and shoot development. In a study on *in-vitro* plant regeneration of soybean cv. BARI-5 using different concentrations of cytokinins and auxins (BAP, 2,4D, and αNAA) individually or in combinations with MS medium, Begum *et al.* (2019) found that the highest shoot formations (shoot induction rate and shoot number) from cotyledonary nodal segments were recorded in MS + 1.5 mg L^{-1} BAP and MS + 0.15 mg L^{-1} BAP + 0.025 mg L^{-1} αNAA. This indicated that a medium with 1.5 mg L^{-1} BA or a combination of BA and αNAA at a low concentration were suitable for shoot induction. In contrast, MS + 0.1 mg L^{-1} IBA and ½ MS + 0.25 mg L^{-1} IBA were optimal for root induction, with the highest average number of roots per shoot and longest root lengths found in MS supplemented with 0.5 mg L^{-1} IBA and 0.1 mg L^{-1} IBA, respectively (Begum *et al.*, 2019).

Effect of BA and αNAA on morphogenesis of calluses in ĐT35 and VNUAĐ2 (Experiment 3)

Calluses obtained in experiment 2 were subcultured on MS + vitamins supplemented with BA and αNAA to induce shoot and root formation. After 6 weeks of culture, the survival rate was 100%, but shoots were induced at low rates in both cultivars (**Table 6**). αNAA inhibited shoot formation while inducing root formation to a certain extent in the presence of BA.

For ĐT35, shoots were induced from calluses, although at a low average rate (2.0%).

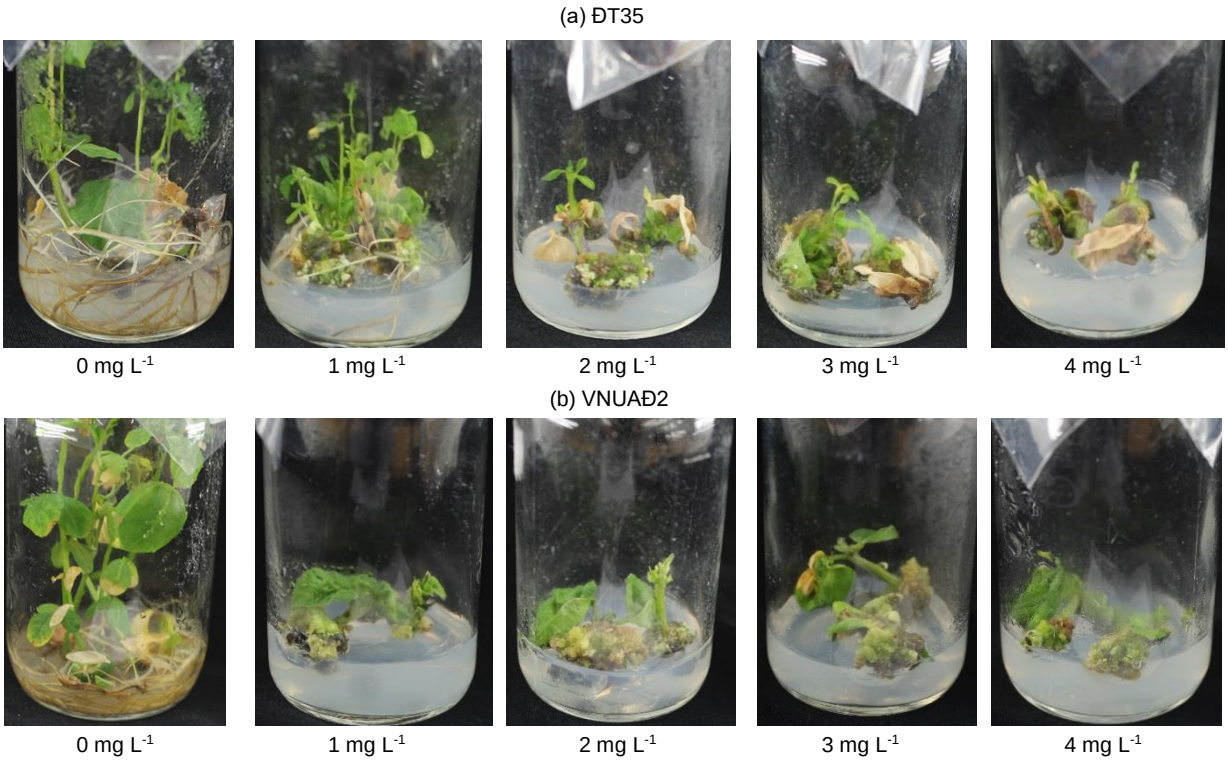


Figure 5. Effect of BA concentration (0, 1, 2, 3, and 4 mg L^{-1}) on morphogenesis from shoot tips 6 weeks after culture of (a) ĐT35 and (b) VNUAĐ2

The highest rate was 7.41% in 1.5 mg L^{-1}BA + 1 mg L^{-1} αNAA but this was not significantly different with 0 and 2 mg L^{-1}BA + 1 mg L^{-1} BA. Generally, calluses formed shoots on MS + 1 mg L^{-1} αNAA supplemented with BA. The root formation rate was highest (25.9%) in the combination with 1.5 mg L^{-1} BA + 0.5 mg L^{-1} αNAA, but the difference was not significant. There were no clear influences of BA and αNAA on the root induction of ĐT35.

In contrast, for VNUAĐ2, no shoots were induced from calluses subcultured on any media supplemented with BA and αNAA. However, root induction was observed, with the highest rate of 44.4% at 0 mg L^{-1} BA + 1 mg L^{-1} αNAA. Under the same αNAA concentration, a high BA concentration inhibited root formation of VNUAĐ2 calluses.

Between the two cultivars, ĐT35 had a higher shoot induction rate (2.1%), but the difference was not statistically significant. Calluses of VNUAĐ2 produced roots at a higher rate than ĐT35 (2.0%), but the difference was not statistically significant. Thus, effects of BA and αNAA on morphogenesis of calluses of ĐT35 and VNUAĐ2 were similar. The rate of shoot production from calluses was low. Effects of αNAA and BA concentrations on root induction were also unclear.

For both cultivars and media without BA, calluses did not grow in size and were dark brown. Media with BA produced green and medium- to large-sized calluses **(Table 7)**. For ĐT35, calluses grew well on MS + 2 mg L^{-1} BA + 0.5 or 1 mg L^{-1} αNAA, while for VNUAĐ2, calluses grew best on MS + 2 mg L^{-1} or 2.5 mg L^{-1} BA + 0.5 mg L^{-1} αNAA, producing greenish and large-sized calluses. Thus, MS + 2 mg L^{-1} BA + 0.5 or 1 mg L^{-1} αNAA could be used to multiply calluses for both cultivars. Shoots in ĐT35 were also small and only formed in MS + 1.5 or 2.0 mg L^{-1} BA + 1 mg L^{-1} αNAA. Roots induced from calluses were generally thin and short. In a study on the regeneration of soybean cultivars DT84 and ĐT22, Do Thi Anh Tuyet (2019) showed that 1.5 mg L^{-1} BA was the most favorable for shoot induction while 1 mg L^{-1} αNAA was the best for root induction. Nguyen Thi Thuy Huong *et al*. (2009) also reported that

Table 6. Effect of BA and αNAA on morphogenesis of calluses of ĐT35 and VNUAĐ2 after 6 weeks of culture

Cultivars	BA (mg L^{-1})	αNAA (mg L^{-1})	No. explants observed	Shoot induction rate (%)	Root induction rate (%)
ĐT35	0	0.5	30	0.0^{a}	23.3^{b-d}
	1.5		27	0.0^{a}	25.9^{cd}
	2		24	0.0^{a}	20.8^{a-d}
	2.5		27	0.0^{a}	11.1^{a-d}
	0	1.0	21	4.8^{ab}	0.0^{ab}
	1.5		27	7.4^{b}	18.5^{a-d}
	2		24	4.2^{ab}	12.5^{a-d}
	2.5		24	0.0^{a}	25.0^{b-d}
VNUAĐ2	0	0.5	30	0.0^{a}	6.7^{a-c}
	1.5		30	0.0^{a}	26.6^{cd}
	2		30	0.0^{a}	33.3^{d}
	2.5		30	0.0^{a}	16.6^{a-d}
	0	1.0	27	0.0^{a}	44.4^{e}
	1.5		27	0.0^{a}	18.5^{a-d}
	2		27	0.0^{a}	0.0^{a}
	2.5		24	0.0^{a}	8.3^{a-c}
Means for cultivar	ĐT35		204	2.0	17.6
	VNUAĐ2		225	0.0	19.6
Means for BA	0		204	1.2^{A}	18.6^{A}
	1.5		225	1.9^{A}	22.4^{A}
	2		108	1.0^{A}	16.7^{A}
	2.5		111	0^{A}	15.3^{A}
Means for αNAA	0.5		105	0	20.6
	1.0		105	2.0	15.9
$LSD_{0.05\ Cultivar}$				2.25	8.34
$LSD_{0.05\ BA}$				3.18	11.8
$LSD_{0.05\ αNAA}$				2.25	8.35
$LSD_{0.05\ Cultivar \times BA \times αNAA}$				6.4	23.7

Note: Values with the same superscript small or capital letters in the same column are not significantly different at 5% probability level. Hyphen (-) is used for significant differences of more than two letters (e.g. a–c = abc; b-d = bcd).

efficient shoot induction in ĐT22 and DT84 was obtained on a medium supplemented with 1.5 mg L^{-1} BA, while rooting induction was obtained on medium with 1 mg L^{-1} IBA.

The appropriate concentrations of PGRs found in this study were in the ranges of other studies. For example, BA concentrations for shoot induction were 1.0-3.0 mg L^{-1} depending on the cultivar, and the αNAA concentration for root induction was 0.5 mg L^{-1}. Dang Trong Luong *et al*. (2013) concluded that 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} αNAA was suitable for shoot induction in DT2008 and ĐT26. Shoot formation increased with a high regeneration frequency of 96.8% and 4.3 shoots in explants with 1.5 mg L^{-1} BA (Soto *et al*., 2013). In addition, Soto *et al*. (2013) identified that 6-day germination period was better than 7- and 8-day germination periods when using cotyledons to produce explants. This study used the middle 7-day germination period to obtain different explants from cotyledons, shoot tips, and hypocotyls. In addition, MS

Table 7. Effect of BA and αNAA on morphology of induced calluses, shoots, and roots of ĐT35 and VNUAĐ2 6 weeks after culture

BA (mg L^{-1})	αNAA (mg L^{-1})	Callus characteristics			Root characteristics≠	Shoot characteristics≠
		Size	Color	Structure		
ĐT35						
0	0.5	Small	Brown	Compact	Small roots ~ 5cm in length	-
1.5	0.5	Medium	Greenish	Compact	-	-
2	0.5	Large	Greenish	Compact	Small roots ~ 5cm in length	-
2.5	0.5	Large	Green with brown	Compact	Small roots ~ 5cm in length	-
0	1	Small	Brown	Compact	-	Shoot cluster
1.5	1	Medium	Green with brown	Compact	Small roots ~ 5cm in length	Small shoots
2	1	Large	Greenish	Compact	Small roots ~ 5cm in length	Small shoots
2.5	1	Large	Greenish	Compact	Small roots ~ 5cm in length	-
VNUAĐ2						
0	0.5	Small	Brown	Compact	-	-
1.5	0.5	Medium	Greenish	Compact	Small roots ~ 3cm in length	-
2	0.5	Large	Greenish	Compact	Small roots	-
2.5	0.5	Large	Greenish	Compact	Small roots	-
0	1	Small	Dark brown	Compact	Well-developed roots	-
1.5	1	Large	Green with brown margin	Compact	White and small roots ~ 3cm in length	-
2	1	Large	Green with brown	Compact	White and small roots ~ 3cm in length	-
2.5	1	Medium	Green with black	Compact	White and small root	-

Note: "-": no root or shoot induction; Callus size: small ≤ 2.5mm, medium 2.5 < ...≤ 5.0mm, and large > 5.0mm.

supplemented with gibberellic acid (GA3) can be used to elongate shoots (Dang Trong Luong *et al*., 2013). Thus, further studies on *in-vitro* regeneration of ĐT35 and VNUAĐ2 to obtain fully developed plantlets are necessary.

Conclusions

The scope of experiments was to find favorable cytokinin and auxin combinations and differentiate values of explant sources for callus and organogenic induction of soybean cultivars. Both GB5 and MS media were efficient in callus induction. Combinations of BA and TDZ or BA and 2,4D inhibited shoot and root induction. Media supplemented with BA produced calluses at a high rate. BA stimulated shoot induction in both shoot tips and cotyledons. However, BA only stimulated shoot formation at appropriate concentrations, and high BA concentrations inhibited shoot formation. As BA concentrations increased, the root induction rate gradually decreased and reached 0% at a concentration of 3.0 mg L^{-1} BA.

The appropriate concentrations of BA for shoot induction for ĐT35 and VNUAĐ2 were about 1.0-2.0 mg L^{-1} and 2.0-3.0 mg L^{-1}, respectively. Shoot tips and cotyledons were more appropriate than hypocotyl segments for soybean regeneration. Calluses and shoots were induced best on MS + 2 mg L^{-1} BA for shoot tip explants and on MS + 3.0 mg L^{-1} BA for cotyledon explants. In addition, MS + 2.0 mg L^{-1} BA + 0.5 or 1.0 mg L^{-1} αNAA can be used to

multiply calluses in ĐT35 and VNUAĐ2. MS + 1.5 or 2.0 mg L^{-1} BA + 1 mg L^{-1} αNAA can be used to induce shoots from calluses in ĐT35. MS + 0.5 mg L^{-1} αNAA is appropriate to induce roots. However, an appropriate medium for VNUAĐ2 calluses to form shoots has not yet been found. Thus, culture medium optimization and standardization through use of MS medium with plant growth regulators like BA, αNAA, and others such as IAA and IBA for soybean regeneration, are recommended.

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