Effects of 6-benziladenine, thidiazuron, and 1-naphthaleneacetic acid on the *in vitro* callus formation and shoot multiplication from leaves of Mai vang (*Ochna integerrima*) HD01 line

Kiet C. Nguyen*, Duy M. Pham, Van H. Phan, Tri M. Bui, & Hau T. Le
Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Nguyen Cao Kiet
Email:
kiet.nguyencao@hcmuaf.edu.vn

ABSTRACT

Mai vang (*Ochna integerrima* (Lour.) Merr.) not only is a popular ornamental plant but also possesses numerous valuable herbal properties. An exceptional line, the HD01, was bred by adept florists at the Binh Loi Mai village, Ho Chi Minh City. The objective of this research was to determine the optimal 6-benziladenine (BA), thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) concentrations appropriate for callus formation from leaf samples and regeneration of shoots from callus of the HD01 line in *in vitro* condition. The study including two experiments was arranged in a completely randomized design with two factors. For callus induction, the culture medium supplemented with concentrations of TDZ combined with concentrations of NAA was used, while to induce shoots, concentrations of BA combined with concentrations of NAA were added to the medium. The results indicated that 0.2 mg/L TDZ combined with 0.25 mg/L NAA on the base of MS (Murashige and Skoog) medium supplemented with 7 g/L agar and 30 g/L sugar gave 100% callus formation rate after 17.6 days, with the largest callus diameter (2.7 cm) and highest callus mass (2.4 g) after 60 days of culture. The leaf-derived callus produced the best results when cultured on MS medium supplemented with 7 g/L agar, 30 g/L sugar, and a combination of 1 mg/L BA and 0.25 mg/L NAA, forming shoot clusters with the height of 2.2 cm, an average of 8.5 shoots per cluster and 9.7 leaves per cluster after 60 days of culture. Based on these results, a preliminary protocol for Mai vang HD01 micropropagation was established.

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1. Introduction

The Mai vang tree (*Ochna integerrima*) is an important ornamental plant in Vietnam. Native to the South East Asia region, the plant is seen as a symbol for the Lunar New Year due to the flowering time usually falling in the holiday. Therefore, *O. integerrima* has become one of the most widely cultivated and traded ornamental plants during the Lunar New Year. Many elite lines and varieties are produced by breeders and growers every year, each with particular superior traits. Among them, the HD01 line selected at Huu Duc mai garden in Binh Loi Mai village has many outstanding characteristics, such as large flower diameter (up to 4 - 6 cm), extended flowering period, and darker petal and stamen color than other lines.

In conventional cultivation, *O. integerrima* is propagated mainly bzy seeds. However, genetic variation makes it difficult to create uniform seedlings, and sometimes the daughter plants no longer retain the value of the parents. Clonal propagation methods such as cutting, air-layering or grafting are also frequently employed for *O. integerrima*, but with low multiplication rates and efficiencies. *In vitro* propagation techniques have the advantage of high propagation rates as well as high uniformity of seedlings, so it has been applied to propagate many plant species with different economic values (Tran, 2005). There have been a few studies on *in vitro* propagation of *O. integerrima* both domestically and internationally, but few have managed to employ cell dedifferentiation and callus formation from leaves to create high propagation rates and utilize the more abundant starting materials. Furthermore, although there has been some success in *in vitro* propagation of *O. integerrima*, the protocols need adjustment, optimizing the mineral contents and plant growth regulators depending on each particular variety or line.

Among the plant growth regulators, auxin and cytokinin play an important role in plant *in vitro* morphogenesis, including callus, shoot and root formation. 6-benziladenine (BA), thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) are common plant growth regulators frequently employed in micropropagation. The combinations of types and concentrations of these plant growth regulators are usually the deciding factor in the morphogenesis and regeneration of plant cell *in vitro*, particularly for *O. integerrima.* Each stage of micropropagation may require very different and specific combinations (Ho et al., 2019). Therefore, the objective of this study was to determine the most appropriate combination for establishing the calluses from leaves of the HD01 line and regeneration of these calluses to shoot clusters.

2. Materials and Methods

2.1. Time and place of the study

The study was carried out from March to July 2023, at the tissue culture lab of the Department of Physiology and Biochemistry, Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam.

2.2. Experimental design

2.2.1. Experiment 1: Effects of TDZ and NAA concentrations on the induction of callus formation from *O. integerrima* leaf samples

Experiment 1 had two factors: factor T (TDZ concentration): 0.1 mg/L; 0.2 mg/L; 0.3 mg/L; 0.4 mg/L, and factor N (NAA concentration): 0 mg/L; 0.25 mg/L; 0.5 mg/L. The experiment consisted of 12 treatments with 3 replications and arranged in a completely randomized design. Each replicate had 5 flasks. Each flask had 3 samples.

2.2.2. Experiment 2: Effects of BA and NAA concentrations on shoot regeneration from callus of *O. integerrima*

Experiment 2 had two factors: factor B (BA concentration): 0 mg/L; 1 mg/L; 2 mg/L; 3 mg/L, and factor N (NAA concentration): 0 mg/L; 0.25 mg/L; 0.5 mg/L. The experiment consisted of 12 treatments with 3 replications and arranged in a completely randomized design. Each replicate had 5 flasks. Each flask had 3 samples.

2.3. Experimental method

The base medium in both experiments was Murashige and Skoog (MS) supplemented with 7 g/L agar, 30 g/L sucrose, and plant growth regulators according to treatments.

In experiment 1, leaves of 8 - 10 cm in length and 4 - 5 cm in width were disinfected with sodium hypochlorite (made in Vietnam) for 20 min and rinsed 3 times with sterilized water. The undamaged sections of the leaves with veins were then cut into 1 × 1 cm and put in the flasks face-down on the medium and kept in the dark for 60 days.

In experiment 2, calluses were produced in large quantity following the best treatment in experiment 1. After 60 days in the dark, the calluses were then put under light (2500 ± 500 lux) for another 10 days. Green, compact and dry calluses were selected, and small samples of these calluses with the diameter of 0.5 cm were dissected and used as starting materials to be transplanted into the flasks for experiment 2. The calluses were grown for 60 days.

2.4. Parameters

2.4.1. Experiment 1

The samples were monitored every day and the day of first callus formation was recorded for each treatment. Colors of the calluses were recorded every 10 days, and the calluses were considered dead when they turned dark brown. On day 60 of culture, the largest diameters (cm) and fresh weights (mg) of 10 random samples were measured. The callus formation rate (%) in each treatment on day 60 was calculated as the number of samples that successfully formed calluses divided by the total number of samples (45). Samples were considered successfully forming callus when undifferentiated, white or yellowish/yellow cell clusters could be observed on the leaf samples.

2.4.2. Experiment 2

The number of shoots and number of leaves per cluster and the height of each shoot cluster (cm) were recorded through the glass layer of the flask every 10 days. Time of shoot formation was defined as the day when more than 50% of the samples formed shoot buds in each treatment. Fresh weights of shoot clusters (mg) were recorded on day 60.

2.5. Data processing and analyses

Data were processed with Excel 2010 (Microsoft, USA). R (version 4.2.3) was used to perform ANOVA and rank the treatments with Duncan's multiple range test at $\alpha = 0.01$ or $\alpha = 0.05$. Data on the rate of callus formation in experiment 1 were transformed using the formula before statistical analysis, the value 0 replaced with 1/4 n and 100 replaced with 100 - 1/4 n (with n = 15 samples) before the formula was applied. Data on the diameter of callus in experiment 1, number of shoots, shoot cluster height and number of leaves in experiment 2 were transformed using the formula before statistical analysis (Ngo et al., 2013).

3. Results and Discussion

3.1. Effect of TDZ and NAA concentrations on the induction of callus from leaf samples of *O. integerrima*

3.1.1. Effect of TDZ and NAA concentrations on the rate and time of callus formation

Table 1. Effect of thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) concentrations on the rate (%) and time (days) of callus formation from leaf samples of *O. integerrima* HD01

	TDZ concentration (mg/L)	NAA concentration (mg/L)			Average (T)
		0	0.25	0.5	
Rate of callus formation (%)	0.1	0	100	93.3	64.4
	0.2	0	100	84.4	61.5
	0.3	0	100	84.4	61.5
	0.4	0	100	82.2	60.7
Average (N)		0^{c}	100^{a}	86.1^{b}	
CV (%) = 17.4%; $F_T = 0.3^{ns}$; $F_N = 299.2^{**}$; $F_{T*N} = 0.3^{ns}$					
Time of callus formation (days)	0.1	-	18.2	17.8	-
	0.2	-	17.6	17.9	-
	0.3	-	17.7	17.8	-
	0.4	-	18.1	18.3	-
Average (N)		-	17.9	18.0	-

*Within the same group, the same letter following the means represents statistically insignificant difference. ns: statistically insignificant difference; **: significant difference at P ≤ 0.01.*

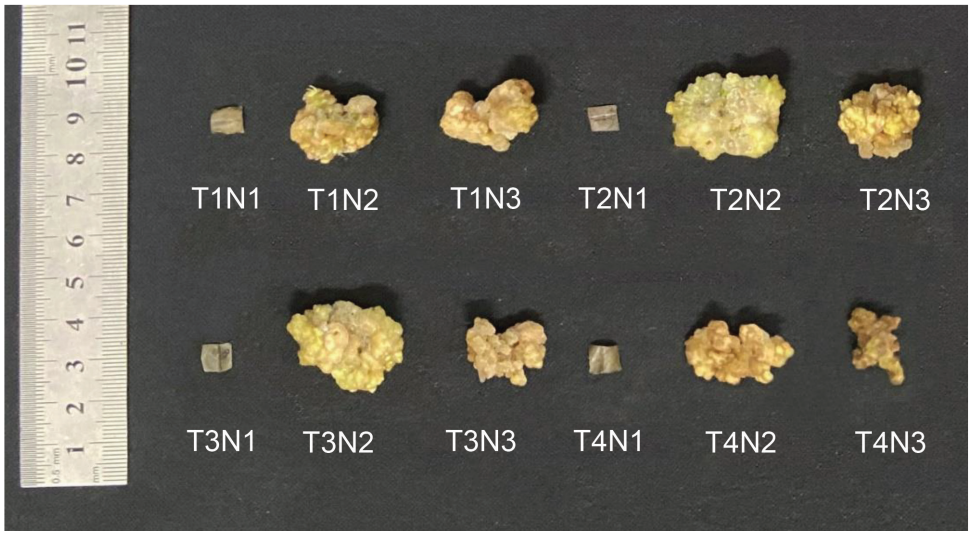

Figure 1. Callus samples of the treatments after 60 days of culture. T1-4: thidiazuron concentration of 0.1, 0.2, 0.3 or 0.4 mg/L, respectively; N1-3: 1-naphthaleneacetic acid concentration of 0, 0.25 or 0.5 mg/L, respectively.

On day 60, the results of Table 1 shows that the TDZ concentration had no effect on the rate of callus formation. However, the NAA concentration significantly affected the rate of callus formation (Figure 1). In particular, the concentration of 0.25 mg/L NAA gave the best result, achieving 100% callus formation rate, significantly higher than the rates of the remaining concentrations. Considering the interaction between TDZ and NAA concentrations, the rate of callus formation was not statistically different, ranging from 82.2% to 100%. The combination of TDZ and NAA has been reported to result in high rates of callus formation in some *in vitro* plants (Verma, 2016; Karakas, 2020). The data of this experiment show similar results. Depending on the type of plants, the most appropriate ratio of TDZ and NAA for callus formation may differ (Lu et al., 2023).

Callus formation time in the treatments ranged from 17.6 days to 18.3 days. In particular, the treatment supplemented with 0.2 mg/L TDZ combined with 0.25 mg/L NAA gave the fastest callus formation time (17.6 days). However, no statistically significant differences were found.

3.1.2. Effect of TDZ and NAA concentrations on color of callus during culture period

Table 2. Effect of thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) concentrations on color of callus from leaf samples of *O. integerrima* HD01

	TDZ concentration (mg/L)	NAA concentration (mg/L)		
		0	0.25	0.5
Day 20	0.1	-	Light yellow	Light yellow
	0.2	-	Light yellow	Light yellow
	0.3	-	Light yellow	Light yellow
	0.4	-	Light yellow	Light yellow
Day 30	0.1	-	Light yellow	Light yellow
	0.2	-	Light yellow	Light yellow
	0.3	-	Light yellow	Light yellow
	0.4	-	Light yellow	Light yellow
Day 40	0.1	-	Yellow	Dark yellow
	0.2	-	Yellow	Dark yellow
	0.3	-	Yellow	Dark yellow
	0.4	-	Yellow	Dark yellow
Day 50	0.1	-	Yellow	Dark yellow
	0.2	-	Yellow	Dark yellow
	0.3	-	Yellow	Dark yellow
	0.4	-	Dark yellow	Dark yellow
Day 60	0.1	-	Dark yellow	Yellow-brown
	0.2	-	Yellow	Golden brown
	0.3	-	Yellow	Pale brown
	0.4	-	Yellow-brown	Brown

As indicated in Table 2, no callus formation was observed in treatments using only TDZ over 60 days of culture. After 20 - 30 days of culture, treatments with combination of TDZ and NAA at any concentrations produced light yellow calluses.

Most distinctions in callus color were observed on day 60, when calluses in all treatments using 0.5 mg/L NAA at any TDZ concentrations showed signs of browning, as well as calluses in treatment with 0.4 mg/L TDZ and 0.25 mg/L NAA which also turned yellow brown. This indicates high concentration of NAA or TDZ induced early aging of the calluses in *O. integerrima*.

3.1.3. Effects of TDZ and NAA concentrations on diameter and fresh weight of callus

Both single factor and interaction between two factors had statistically significant effects on diameter and fresh weight of callus. Regarding the interaction between these two factors (Table 3), at the NAA concentration of 0.25 mg/L, both diameter and fresh weight of callus were improved as TDZ concentration increased from 0.1 mg/L to 0.2 or 0.3 mg/L. Interestingly, at the NAA concentration of 0.5 mg/L, no significant change in diameter and fresh weight of callus when TDZ concentration changed was observed. This could indicate that the effect of NAA at this concentration was too strong and masking the effect of TDZ.

Table 3. Effects of thidiazuron (TDZ) and 1-naphthaleneacetic acid (NAA) concentrations on diameter (cm) and fresh weight (g) of *O. integerrima* HD01 samples after 60 days of culture

	TDZ concentration (mg/L)	NAA concentration (mg/L)			Average (T)
		0	0.25	0.5	
Callus diameter (cm)	0.1	0^{c}	1.8^{b}	1.9^{b}	1.2^{ab}
	0.2	0^{c}	2.7^{a}	1.8^{b}	1.5^{a}
	0.3	0^{c}	2.6^{a}	1.8^{b}	1.5^{a}
	0.4	0^{c}	1.9^{b}	1.6^{b}	1.2^{b}
Average (N)		0^{c}	2.3^{a}	1.8^{b}	
CV = 5.5%; $F_T = 4.1^{*}$; $F_N = 618.3^{**}$; $F_{T*N} = 3.9^{**}$					
Fresh weight of samples (g)	0.1	0^{c}	1.4^{b}	1.4^{b}	1.0^{b}
	0.2	0^{c}	2.4^{a}	1.4^{b}	1.3^{a}
	0.3	0^{c}	2.4^{a}	1.3^{b}	1.2^{a}
	0.4	0^{c}	1.5^{b}	1.2^{b}	0.9^{b}
Average (N)		0^{c}	1.9^{a}	1.3^{b}	
CV (%) = 11.6%; $F_T = 20.9^{**}$; $F_N = 731.6^{**}$; $F_{T*N} = 19.7^{**}$					

*Within the same group, the same letter following the means represents statistically insignificant difference. *: significant difference at 0.01 < P ≤ 0.05; **: significant difference at P ≤ 0.01.*

On the other hand, the leaf samples all failed to form any callus when no NAA was supplemented, regardless of the TDZ concentration. For the formation of callus in many plant species, auxin and cytokinin concentrations need to be at a certain balance (Ikeuchi et al., 2013). The interaction between auxin and cytokinin is especially important to control several processes of formation, development and maintenance of meristems necessary to create the plant body (Su et al., 2011). Nitsch (1986) asserted that only the simultaneous action of auxin and cytokinin can strongly stimulate DNA synthesis and induce cell division. While TDZ has been reported to exhibit

both cytokinin and auxin effects, the cytokinin activity is still much more potent, which is why TDZ is still widely considered a cytokinin (Ahmad & Faisal, 2018). This could explain why using TDZ alone was shown to be insufficient to generate callus in *O. integerrima* in this study.

3.2. Effects of the combination of BA and NAA on shoot regeneration from callus of *O. integerrima* HD01

3.2.1. Effects of BA and NAA concentrations on the number of shoots generated from callus

Table 4 shows the number of shoots in the shoot clusters generated from calluses of *O. integerrima* during the culture period of 60 days. On day 10 - 20, the shoot buds were too small to be distinguished accurately with naked eyes, therefore these data were omitted.

Table 4. Effects of 6-benziladenine (BA) and 1-naphthaleneacetic acid (NAA) concentrations on the number of shoots (shoots/cluster) generated from callus of *O. integerrima* HD01

Days of culture	BA concentrations (mg/L)	NAA concentrations (mg/L)			Average (B)
		0	0.25	0.5	
30	0	0^{d}	0.4^{cd}	0.5^{c}	0.3^{c}
	1	3.7^{b}	7.9^{a}	3.3^{b}	4.9^{a}
	2	2.9^{b}	3.3^{b}	0.2^{cd}	2.1^{b}
	3	3.0^{b}	3.1^{b}	0.8^{cd}	2.3^{b}
Average (N)		2.4^{b}	3.7^{a}	1.21^{b}	
CV (%) = 6.4%; $F_B = 311.4^{**}$; $F_N = 183.0^{**}$; $F_{B*N} = 42.8^{**}$					
40	0	0^{e}	0.4^{d}	0.5^{d}	0.3^{c}
	1	4.1^{b}	8.3^{a}	3.5^{bc}	5.3^{a}
	2	3.0^{c}	3.6^{bc}	0.3^{de}	2.3^{b}
	3	3.0^{c}	3.3^{bc}	0.3^{de}	2.2^{b}
Average (N)		2.5^{b}	3.88^{a}	1.13^{c}	
CV (%) = 5.8%; $F_B = 385.2^{**}$; $F_N = 221.5^{**}$; $F_{B*N} = 47.9^{**}$					
50	0		0.5^{c}	0.5^{c}	0.3^{c}
	1		8.3^{a}	3.6^{b}	5.4^{a}
	2		3.8^{b}	0.4^{c}	2.5^{b}
	3		3.7^{b}	0.3^{c}	2.4^{b}
Average (N)			4.1^{a}	1.2^{c}	
CV (%) = 8.2%; $F_B = 186.5^{**}$; $F_N = 108.7^{**}$; $F_{B*N} = 23.8^{**}$					
60	0	0^{d}	0.5^{cd}	0.7^{c}	0.4^{c}
	1	4.3^{b}	8.5^{a}	3.7^{b}	5.5^{a}
	2	3.3^{b}	3.9^{b}	0.5^{cd}	2.6^{b}
	3	3.2^{b}	3.8^{b}	0.4^{cd}	2.5^{b}
Average (N)		2.7^{b}	4.2^{a}	1.3^{c}	
CV (%) = 8.1%; $F_B = 177.8^{**}$; $F_N = 98.0^{**}$; $F_{B*N} = 23.6^{**}$					

*Within the same group, the same letter following the means represents statistically insignificant difference; **: significant difference at $P \leq 0.01$.*

The results show that as early as day 30, when the shoot buds were distinguishable, significant differences among treatments due to the effects of each factor and the interaction between two factors could be observed, and these differences maintained until the end of the culture period (day 60). The numbers of shoots only slightly increased in all treatments from day 30 to day 40, with the largest increase in treatment with 1 mg/L BA and no NAA (from 3.7 to 4.1 shoots, equivalent to a 10.8% increase), and beyond day 40, they remained largely unchanged. This indicates that the reprograming of different *O. integerrima* callus cells to shoot meristems happened simultaneously soon after the exposure to the change in the growth regulator environment. Maintaining the growth regulators might still be necessary for the development of these shoots, but very few new shoot meristems were formed in the later half of the culture period. This information may be useful for future studies on the cellular and molecular mechanisms of the cell morphogenesis in *O. integerrima* under the effects of plant growth regulators.

The trend in the difference of number of shoots among treatments was also largely the same throughout the culture period. When no BA was used, very few shoots were formed regardless of the NAA concentration, which demonstrates the important role of BA in reprograming the unorganized callus cells into shoot meristems that could develop into shoot buds. At high concentrations of both BA (2 or 3 mg/L) and NAA (0.5 mg/L), the *O. integerrima* calluses also generate very low number of shoots, which suggests that these concentrations had become excessive for *O. integerrima* cells and inhibited the regeneration of shoots. The remaining combinations of BA and NAA concentrations resulted in statistically similar number of shoots regenerated from the calluses, except for the combination of 1 mg/L BA and 0.25 mg/L NAA, which gave the highest number of shoots regenerated (8.5 shoots/cluster on day 60), statistically higher than all other treatments (Figure 2). These results are consistent with the research of Ho et al. (2019) on stem cuttings, which showed the combination of 1.5 mg/L BA and 0.5 mg/L NAA achieved the highest number of shoots (3.4 shoots) regenerated from the original culture. Compared with the results of Mai & Lam (2013), the shoot multiplication rate in this study was much higher (8.5 compared to 2.3), which proves that the combination of BA and NAA gives better results than using BA alone.

Figure 2. *In vitro O. integerrima* shoots after 60 days of culture in different combinations of 6-benziladenine (BA) and 1-naphthaleneacetic acid (NAA) concentrations. B1-4: BA concentration of 0, 1, 2 or 3 mg/L, respectively; N1-3: NAA concentration of 0, 0.25 or 0.5 mg/L, respectively.

3.2.2. Effect of BA and NAA concentrations on the height and number of leaves of the shoot cluster generated from callus

Unlike the number of shoots, the height of shoot cluster, which represents the growth of shoots, shows gradual increase in some treatments, specifically treatments with 1 mg/L BA at all NAA concentrations, as well as treatment with 2 mg/L BA combined with 0.25 mg/L NAA, over the culture period of 60 days (Table 4). In the rest of the treatments, very low increase in shoot cluster height was observed, indicating that very little growth of the shoots happened in these treatments.

Table 5. Effects of 6-benziladenine (BA) and 1-naphthaleneacetic acid (NAA) on shoot cluster height (cm) of *O. integerrima* HD01

Days of culture	BA concentration (mg/L)	NAA concentration (mg/L)			Average (B)
		0	0.25	0.50	
30	0	0^{c}	0^{c}	0^{c}	0^{b}
	1	0.8^{ab}	1.2^{a}	0.5^{abc}	0.8^{a}
	2	0^{c}	0.4^{bc}	0^{c}	0.1^{b}
	3	0.1^{c}	0.1^{c}	0^{c}	0.1^{b}
Average (N)		0.8^{b}	0.4^{a}	0.13^{b}	
CV (%) = 13.9%; $F_B = 22.8^{**}$; $F_N = 5.0^{*}$; $F_{B*N} = 1.1^{ns}$					
40	0	0^{d}	0^{d}	0^{d}	0^{b}
	1	1.0^{ab}	1.4^{a}	0.7^{abc}	1.0^{a}
	2	0^{d}	0.6^{bc}	0^{d}	0.2^{b}
	3	0.2^{cd}	0.3^{cd}	0^{d}	0.2^{b}
Average (N)		0.3^{b}	0.6^{a}	0.2^{b}	
CV (%) = 13.1%; $F_B = 30.5^{**}$; $F_N = 7.4^{**}$; $F_{B*N} = 1.6^{ns}$					
50	0	0^{c}	0^{c}	0.1^{c}	0^{c}
	1	1.3^{b}	1.9^{a}	1.08^{b}	1.5^{a}
	2	0^{c}	0.9^{b}	0.05^{c}	0.3^{b}
	3	0.2^{c}	0.3^{c}	0.04^{c}	0.2^{bc}
Average (N)		0.4^{b}	0.8^{a}	0.3^{b}	
CV (%) = 11.0%; $F_B = 69.6^{**}$; $F_N = 14.9^{**}$; $F_{B*N} = 3.6^{*}$					
60	0	0^{c}	0.1^{c}	0.1^{c}	0^{c}
	1	1.4^{b}	2.2^{a}	1.2^{b}	1.6^{a}
	2	0.0^{c}	0.9^{b}	0.1^{c}	0.3^{b}
	3	0.3^{c}	0.3^{c}	0.1^{c}	0.2^{bc}
Average (N)		0.4^{b}	0.9^{a}	0.4^{b}	
CV (%) = 9.8%; $F_B = 95.9^{**}$; $F_N = 19.9^{**}$; $F_{B*N} = 4.5^{**}$					

*Within the same group, the same letter following the means represents statistically insignificant difference; ns: nonsignificant difference; *: significant difference at 0.01 < P ≤ 0.05; **: significant difference at P ≤ 0.01.*

Despite the gradual increase in shoot cluster height over the culture period, the trend in the differences among treatments remained similar from day 30 to 60. The BA concentration of 1 mg/L was clearly the most appropriate for shoot growth, as at any NAA concentration, the treatment with 1 mg/L BA gave the highest results statistically compared to other BA concentrations. Among treatments with 1 mg/L BA, the combination with 0.25 mg/L NAA also give the highest results of cluster height (2.2 cm on day 60, equivalent to a 57.1% higher than the second-best treatment of 1 mg/L BA and no NAA), which coincides with the best number of shoots in this treatment, showing that this combination is the most appropriate for generation of shoots from *O. integerrima* leaf callus. Cytokinin stimulates shoot formation directly or indirectly on intact plants as well as on *in vitro* cultured plant tissue. This is more effective when combining cytokinin with auxin in an appropriate ratio (Nguyen & Le, 2006).

Table 6. Effects of 6-benziladenine (BA) and 1-naphthaleneacetic acid (NAA) concentrations on the number of leaves formed during shoot regeneration of *O. integerrima* HD01

Days after implantation	BA concentrations (mg/L)	NAA concentrations (mg/L)			Average (B)
		0	0.25	0.5	
30	0	0.0^{f}	0.4^{de}	0.2^{ef}	0.2^{c}
	1	1.5^{abc}	2.3^{a}	1.4^{bc}	1.8^{a}
	2	0.4^{ef}	2.2^{ab}	0.3^{ef}	1.0^{bc}
	3	1.3^{bc}	1.3^{cd}	0.4^{ef}	1.0^{b}
Average (N)		0.8^{b}	1.5^{a}	0.6^{b}	
CV (%) = 10.3%; $F_B = 39.8^{**}$; $F_N = 39.2^{**}$; $F_{B*N} = 6.9^{**}$					
40	0	0.0^{d}	0.2^{d}	0.1^{d}	0.1^{c}
	1	4.2^{b}	9.0^{a}	4.6^{b}	5.9^{a}
	2	0.1^{d}	7.5^{a}	0.2^{d}	2.6^{b}
	3	2.7^{c}	2.2^{c}	0.3^{d}	1.8^{b}
Average (N)		1.8^{b}	4.7^{a}	1.3^{b}	
CV (%) = 9.5%; $F_B = 211.2^{**}$; $F_N = 123.5^{**}$; $F_{B*N} = 42.5^{**}$					
50	0	0.0^{d}	0.2^{d}	0.1^{d}	0.1^{c}
	1	4.4^{bc}	9.6^{a}	4.7^{bc}	6.2^{a}
	2	0.1^{d}	7.6^{ab}	0.2^{d}	2.6^{b}
	3	2.9^{c}	2.3^{c}	0.3^{d}	1.8^{b}
Average (N)		1.8^{b}	4.9^{a}	1.3^{c}	
CV (%) = 10.5%; $F_B = 177.4^{**}$; $F_N = 111.1^{**}$; $F_{B*N} = 35.5^{**}$					
60	0	0.0^{d}	0.3^{d}	0.2^{d}	0.2^{c}
	1	4.4^{b}	9.7^{a}	4.8^{b}	6.3^{a}
	2	0.2^{d}	7.7^{a}	0.3^{d}	2.7^{b}
	3	3.0^{c}	2.3^{c}	0.3^{d}	1.9^{b}
Average (N)		1.9^{b}	5.0^{a}	1.4^{b}	
CV (%) = 10.0%; $F_B = 185.9^{**}$; $F_N = 115.2^{**}$; $F_{B*N} = 36.9^{**}$					

*Within the same group, the same letter following the means represents statistically insignificant difference; **: significant difference at P ≤ 0.01.*

Data on the number of leaves (Table 5) also show that there were few changes in the differences among treatment from day 30 to 60, indicating that the effects of the growth regulator combinations were largely determined early in the culture period. This is despite the fact that the number of leaves still increased substantially from day 30 to day 40.

The BA concentration of 1 mg/L was also shown to be most appropriate for the development of *O. integerrima* shoots from callus, indicated by the highest number of leaves at this concentration compared to other BA concentrations within the same NAA concentration, although at the NAA concentration of 0.25 mg/L, there was no significant difference between 1 mg/L and 2 mg/L BA (Table 6). Leaves affect by these two treatments also gave the highest number (9.7 and 7.7 leaves on day 60, respectively).

Collectively, the results obtained show that the combination of 1 mg/L BA and 0.25 mg/L NAA is most appropriate for the rapid *in vitro* regeneration of *O. integerrima* shoots from leaf callus. The shoot clusters in this treatment had high number of shoots and developed well, contributing to a high propagation rate.

4. Conclusions

For the callus formation from leaf sample, the combination of 0.2 mg/L TDZ and 0.25 mg/L NAA gave the best results with a callus formation rate of 100%, the highest callus diameter and fresh weight, reaching 2.7 cm and 2.4 g, respectively, after 60 days of culture. The calluses remained yellow on day 60 of culture.

For the shoot regeneration from the calluses, using MS medium supplemented with 1 mg/L BA combined with 0.25 mg/L NAA gave shoot clusters with the highest number of shoots (8.5 shoots), cluster height (2.2 cm) and number of leaves (9.7 leaves) after 60 days of culture.

Conflict of interest

The authors have no conflicts of interest to declare.

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