EFFECTS OF PLANT GROWTH REGULATORS ON THE SHOOT MULTIPLICATION AND ROOT FORMATION OF *Violar tricolor* L.

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ABSTRACT

Violar tricolor belongs to Viola genus, *Violaceae* family, Violar tricolor plant is not only grown as an ornamental plant, but also a medicinal herb. In this study, some of factors affect on the process of micropropagation were investigated. In the experiment of sterilization explants, seed germination and shoot formation ability of the *Violar tricolor* L. reached the best result when they were sterilized by 0.1% $HgCl_2$ for 1 minute. In the experiment studying effects of individual BA or BA in combination with NAA on the induction of formation and multiplication shoot, the results showed that shoot cultured in the MS medium supplemented with 0.5 mg/L BA get the highest number of shoots (4.83 shoots), shoots were vigorous without mutation and hyperhydricity. The MS medium containing 0.1 mg/L NAA induced the root formation with numerous long roots and vitality shoots.

Keywords: BA, $HgCl_2$, NAA, *Violar tricolor* L.

1. INTRODUCTION

Violar tricolor belongs to Viola genus, Violaceae family. There are other names such as pansy, or pensée. *Violar tricolor* has been imported into our country since the early years of the 20th century, planted in Da Lat, Ha Noi and many other big cities. These plants are grown in decorative forms in pots and flowerbeds, walkways to flower gardens or parks. Besides, the *Violar tricolor* plant is grown as an ornamental plant; it is also a medicinal herb. *Violar tricolor* is a medicinal herb in Europe and officially recorded in European Franciscan - Copoeia (European pharmacopoeia). In folk medicine, *Violar tricolor* is believed to have soothing, cleansing and anti-inflammatory properties, which are also effective for treating skin diseases such as eczema, psoriasis and acne. Flowers of *Violar tricolor* contain mucus that helps relieve coughs and wheezing due to asthma. These flowers also help fight swelling in rheumatoid and gout cases, helping to lower blood pressure and cholesterol. The number of *Violar tricolor* cyclotides such as Vitri A, Vitri A varv A and Vitri varv E... have toxic activity, kill cancer cells when testing on the cancer cell lines U-937 GTB (lymphoma) and RPMI-8226/s (myeloma) [1].

There are many studies about the cultivation, inheritance and breeding of pansy [2]. Babber and Kulbhushan (1991) successfully induced callus derived from root, hypocotyls and cotyledonary segments of *Viola tricolor* [3]. Sato *et al.* (1995) achieved regeneration of

plantlets from petiole callus of wild viola (*Viola patrinii* DC.) [4]. Wijowska *et al*. (1999) obtained callus, autonomous endosperm and roots *in vitro* by culturing unfertilized ovules of *Viola odorata* [5]. Jian and Ma (2006) were successful in plant regeneration from callus of pansy [6]. Meng *et al*. (2010) studied pansy, the results showed that the WPM medium supplemented with 1.5-2.5 mg/L BA and 0.1 mg/L NAA was suitable for shoot multiplication [7].

In the present investigation, the plant growth regulators influencing the induction and shoot multiplication and root formation *in vitro* on *Violar tricolor* were surveyed.

2. MATERIALS AND METHODS

2.1. Plant material

Explants of this study are *Viola tricolor* seeds, which were brought from Tan Nong Phat Seed Co., Ltd.

2.2. Methods

2.2.1. Seed germination and shoot formation of V. tricolor

Seeds were sterilized with alcohol 70% for 30 seconds. Continuously, they sterilized with $HgCl_2$ 0.1% and Tween 20 with different time periods such as 1, 2, 3, 4, 5 minutes in order to estimate appropriate time for sterilizing. Disinfected seeds were cultured in MS medium [8] supplemented with 30 g/L sucrose, 8 g/L agar, without plant growth regulators. The experiment design is completely randomized design (CRD), one factor with 3 replications, each repeated 10 flasks, 9 explants/flask.

The rate of germinated explants (%), the rate of infected explants (%) were collected after 2 weeks of culture.

2.2.2. Effect of BA individual on shoot proliferation of V. tricolor

Shoots derived from the seeds were excised (1.5 cm of height) and cultured in MS medium supplemented with 30 g/L sucrose, 8 g/L agar and BA at different concentration levels (0.5; 1.0; 1.5; 2.0; 2.5 mg/L). The experiment design is completely randomized design (CRD); one factor with 3 replications, each repeated 10 flasks, 3 explants/flask.

The number of shoots, height of shoot (cm) and numbers of leaf were collected after 4 weeks of culture.

2.2.3. Effect of BA in combination with NAA on shoot proliferation of V. tricolor

Shoots derived from the seeds were excised (1.5 cm of height) and cultured in MS medium supplemented with 30 g/L sucrose, 8 g/L agar and BA (the best concentration for shoot proliferation) combination with NAA (0.5; 1.0; 1.5; 2.0; 2.5 mg/L). The experiment design is completely randomized design (CRD); one factor with 3 replications, each repeated 10 flasks, 3 explants/flask.

The number of shoots, height of shoot (cm), and number of leaf were collected after 4 weeks of culture.

2.2.4. Effect of NAA on root formation of V. tricolor

Shoots (1.5 cm of height) were cultured in MS medium containing 30 g/L sucrose, 8 g/L agar and NAA at different concentration (0.5; 1.0; 1.5; 2.0 mg/L). The experiment design is completely randomized design (CRD); one factor with 3 replications, each repeated 10 flasks, 3 explants/flask.

The rate of root formation explant (%), number of roots, height of shoot (cm), and length of root (cm) were collected after 4 weeks of culture.

2.3. Cultural conditions

All media were autoclaved (121 °C at 1 atm for 20 min) after adjustment of the pH 5.7-5.8. All growth stages of this study were incubated under conditions: 25 ± 2°C, 60 ± 5% relative humidity and a 12-h photoperiod under a photosynthetic photon flux density of 40 ± 5 μmol.m^{-2} s^{-1}.

2.4. Statistical analysis

$$\text{The rate of germinated seeds (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

$$\text{The rate of infected explant (\%)} = \frac{\text{Number of infected explants}}{\text{Total number of explant}} \times 100$$

$$\text{The rate of root formation explant (\%)} = \frac{\text{Number of root formation explants}}{\text{Total number of explant}} \times 100$$

Data were test by Duncan's multiple range test at 5% level using SPSS (version 16.0) software package.

3. RESULTS AND DISCUSSION

3.1. Seed germination and shoot formation ability

The procedure and time of sterile have a significant influence on the germination and vitality of seeds. After two weeks of culture, the effect of sterilization time by $HgCl_2$ 0.1% on seed germination ability was evaluated. Experimental results are shown in Table 1.

Table 1. Effect of time of sterilization on the rate of germinated seeds *Violar tricolor* L.

Time (min)	The rate of infected explant (%)	The rate of germinated explant (%)
1	0	65.1^{a}
2	0	51.4^{ab}
3	0	48.4^{bc}
4	0	37.0^{bc}
5	0	33.3^{b}
F	ns	*
CV (%)		20.0

(*): Mean in the same columns that are followed by different letters is significantly different ($p \leq 0.05$) using Duncan's multiple range tests.

After 2 weeks of seeding, seeds of the experiment germinated and most of them were not contaminated. However, different disinfection times responded to different germination rates. The germination rate was inversely proportional to the sterilization time. The seed decontamination time of *V. tricolor* with $HgCl_2$ 0.1% for 1.0 minute resulted the highest

germination rate of 65.18%, vigorous seedlings and being a beneficial material for the next step of proliferation. Thus, the longer sterilization time, the more injury the seeds were, and resulting in the low sprout.

3.2. Effect of BA individual on shoot multiplication of *V. tricolor in vitro*

After 4 weeks of culture, all treatments had shoot formation. However, the number and vitality of shoots was different in diverse concentration of BA (Table 2).

Table 2. Effect of BA on shoot proliferation of *V. tricolor* after 4 weeks of culture

BA (mg/L)	Number of shoots/explant	Shoot length (cm)	Number of leaves
0.0	1.33^{c}	3.03^{a}	4.07^{b}
0.5	4.83^{a}	2.32^{b}	5.90^{a}
1.0	3.80^{ab}	1.80^{bc}	3.33^{b}
1.5	3.30^{b}	1.97^{bc}	4.53^{ab}
2.0	2.85^{b}	1.70^{bc}	3.40^{b}
2.5	3.17^{b}	1.50^{c}	4.20^{b}
F	**	**	*
CV (%)	22.85	16.29	20.43

(*) Mean in the same columns that are followed by different letters is significantly different ($p \leq 0.05$), (**) significantly different ($p \leq 0.01$) using Duncan's multiple range tests.

The highest numbers of shoots (4.83 shoots) in the medium MS supplemented with 0.5 mg/L BA were vigorous, but when the BA concentration increased, the number of shoots decreased. In high concentrations of BA, there were several mutation shoots, twisted and dark green leaves, vitrification phenomenon. Excessive BA concentration would form shoot clusters, mutation and hyperhydricity. In the study about *Jatrophar curcas*, Do Dang Giap et al. (2012) demonstrated that MS medium supplemented with 0.5 mg/L BA was suitable for shoot formation and proliferation, the rate of formation shoot reached 100% and the number of formation shoot got 8.67 shoot/explants [9]. According to Meng *et al*. (2010) [7], MS medium supplemented with 1.5-2.5 mg/L BA, 0,1 mg/L NAA is suitable for pansy shoot multiplication. Micropropagation of *Thymus piperella* was reported BA stimulated shoot proliferation of explants. With the increase in BA level (0.0-1.5 mg/L), the number of shoots increased [10].

The height of shoot was highest in MS medium without BA, when the medium supplemented with BA, the height's shoots were lower and some shoots were mutation, except for the case of the medium with low BA concentration (0.5 mg/L BA). For *Dendrocalamus asper*, shoots cultured in the PGR-free medium were higher than shoots in MS medium supplemented with BA [11]. Besides, the number of leaves in the medium with 0.5 mg/L BA reached the highest for 5.90 leaves. Thus, MS medium supplemented with BA 0.5 mg/L was suitable for the proliferation ability, the number of shoots was 4.83, shoots were healthy and not mutated.

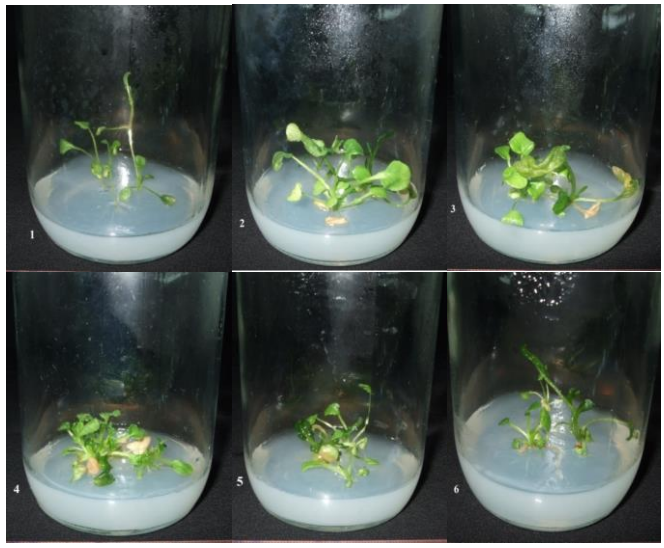

Figure 1. Effect of BA on shoot proliferation of *V. tricolor* after 4 weeks
1) 0 mg/L BA; 2) 0.5 mg/L BA; 3) 1.0 mg/L BA; 4) 1.5 mg/L BA, 5) 2.0 mg/L BA; 6) 2.5 mg/L BA.

3.3. Effect of BA and NAA on the growth and proliferation shoot of *V. tricolor*

All treatments obtained shoot formation with 100% shoot multiplication rate after 4 weeks of culture. However, in medium containing 0.5 mg/L BA combination with different NAA concentrations, the number of axillary shoots formation was different (Table 3).

Table 3. Effect of BA in combination with NAA on shoot proliferation of *V. tricolor* after 4 weeks of culture

Treatment	BA (mg/L)	NAA (mg/L)	Number of shoots	Shoot length (cm)	Number of leaves
1	0.0	0.0	1.01^{c}	3.90^{a}	6.13^{b}
2	0.5	0.1	1.20^{bc}	3.00^{b}	5.37^{cd}
3	0.5	0.2	1.40^{b}	3.12^{b}	5.80^{bc}
4	0.5	0.3	1.23^{bc}	3.38^{b}	5.40^{cd}
5	0.5	0.4	2.10^{a}	3.41^{b}	7.03^{a}
6	0.5	0.5	1.17^{bc}	3.20^{b}	5.21^{d}
F			**	*	**
CV (%)			13.4	7.6	5.0

(*) Mean in the same columns that are followed by different letters are significantly different ($p \leq 0.05$), (**) significantly different ($p \leq 0.01$) using Duncan's multiple range tests.

On MS medium supplemented NAA with gradually increasing concentration from 0.1 mg/L to 0.4 mg/L in combination with 0.5 mg/L BA, the number of shoots formation increased gradually; but when the concentration of NAA reached 0.5 mg/L, the number of shoots was reduced. Treatment 5 (medium containing 0.5 mg/L BA and 0.4 mg/L NAA) attained the highest number of shoots (2.1 shoots) and leaves (7.03 leaves). On PGR-free medium, shoots regenerated for shoot stem prolongation with mean length of 3.9 cm were obtained.

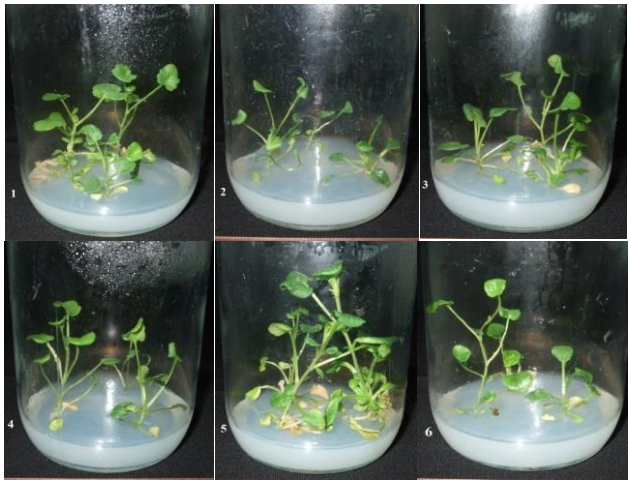

Figure 4. Effect of BA in combination with NAA on shoot proliferation of *V. tricolor* after 4 weeks
1) Control; 2) 0.5 mg/L BA + 0.1 mg/L NAA; 3) 0.5 mg/L BA + 0.2 mg/L NAA;
4) 0.5 mg/L BA + 0.3 mg/L NAA; 5) 0.5 mg/L BA + 0.4 mg/L NAA; 6) 0.5 mg/L BA + 0.5 mg/L NAA.

3.4. Effect of NAA on root formation of V. tricolor shoot

The best rooting potential of shoots was achieved when shoots were cultured on medium MS supplemented with 0.1 mg/L NAA. The highest number of roots in treatment 2 (0.1 mg/L NAA) was 5.30 roots (Table 4). Essentially, when the concentration of NAA increased, it excited the roots formation; then the number of roots gradually decreased whether NAA concentration increased. Therefore, NAA stimulates root formation, but high concentrations of NAA inhibited root formation of *Violar tricolor* L.

Table 4. Effect of NAA on root formation of *Violar tricolor* shoot after 4 weeks of culture

Treatment	NAA (mg/L)	Formation root rate (%)	Number of roots	Root length (cm)	Shoot height (cm)
1	0.0	100	4.17^{b}	0.35^{b}	6.20^{a}
2	0.1	100	5.30^{a}	0.50^{a}	5.21^{ab}
3	0.5	100	3.83^{b}	0.32^{bc}	4.43^{b}
4	1.0	100	3.23^{bc}	0.42^{ab}	5.03^{ab}
5	1.5	100	2.80^{c}	0.21^{c}	4.53^{b}
6	2.0	100	1.80^{d}	0.33^{bc}	4.10^{b}
F		ns	**	**	*
Cv(%)			15.18	19.86	15.10

(*) Mean in the same column that are followed by different letters are significantly different ($p \leq 0.05$), (**) significantly different ($p \leq 0.01$), and (ns) no significant using Duncan's multiple range test.

The MS medium with 0.1 mg/L NAA resulted in the highest root length, which reached 0.50 cm, but when the NAA increased to a high level of concentration, the root length decreased. On the medium without NAA, plantlets prolonged their height and reached the highest length (6.20 cm). Therefore, the MS medium supplemented with 0.1 mg/L NAA was suitable for the root formation of the *Violar tricolor* L., reached several long roots (5.30 roots), propagules were tall, green and vigorous. Jian and Ma (2006) also demonstrated that NAA was suitable for root induction of pansy [6].

Figure 4. Effect of NAA on root formation of *V. tricolor* shoot after 4 weeks
1) Control; 2) 0.1 mg/L NAA; 3) 0.5 mg/L NAA; 4) 1.0 mg/L NAA; 5) 1.5 mg/L NAA; 6) 2.0 mg/L NAA.

4. CONCLUSION

In this study, seed germination and shoot formation ability of *Violar tricolor* L. reached the best result when seeds were sterilized by 0.1% $HgCl_2$ for 1 minute and got the best germination without contamination. In the experiment of the effect of medium containing individual BA or BA in combination with NAA on the induction of shoot formation and multiplication, MS medium supplemented with 0.5 mg/L BA showed the best result of shoot proliferation with the highest multiplication rate (100%), the number of shoots (4.83 shoots); shoots were vigorous without mutation and hyperhydricity; besides, on MS medium supplemented with 0.5 mg/L BA and 0.4 mg/L NAA, most of shoots were healthy. The MS medium containing 0.1 mg/L NAA induced the root formation with numerous long roots and vitality shoots.

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TÓM TẮT

ẢNH HƯỞNG CỦA CHẤT ĐIỀU HÒA SINH TRƯỞNG THỰC VẬT LÊN SỰ NHÂN NHANH CHỒI VÀ TẠO RỄ CỦA CÂY HOA BƯỚM (*Violar tricolor* L.) NUÔI CẤY *IN VITRO*

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Cây hoa bướm (*Violar tricolor*), thuộc chi Viola, họ Violaceae, bộ Violase, là loài hoa đẹp không những trồng để trang trí nhà, vườn hoa hay công viên mà còn được xem là một loài dược liệu. Trong nghiên cứu này, một số yếu tố ảnh hưởng đến quá trình vi nhân giống cây hoa bướm được khảo sát. Kết quả nghiên cứu cho thấy, khử trùng hạt cây hoa bướm (*Violar tricolor* L.) với $HgCl_2$ 0,1% trong thời gian 1 phút cho kết quả tốt nhất với tỷ lệ nảy mầm cao và mẫu không bị nhiễm. Trong thí nghiệm khảo sát ảnh hưởng của BA riêng lẻ hay BA kết hợp với NAA lên khả năng cảm ứng và nhân chồi của loài cây này, kết quả cho thấy môi trường thích hợp hình thành chồi cây hoa bướm (*Violar tricolor* L.) là môi trường MS có bổ sung BA với nồng độ 0,5 mg/L cho tỷ lệ hình thành chồi cao, số chồi nhiều (4,83 chồi), chồi to, khỏe không bị biến dị và thủy tinh thể. Môi trường MS có bổ sung 0,1 mg/L NAA thích hợp cho sự hình thành rễ của cây hoa bướm (*Violar tricolor* L.).

Từ khóa: BA, hoa bướm, $HgCl_2$, NAA.