

IN VITRO ADVENTITIOUS SHOOT REGENERATION FROM COTYLEDON EXPLANT OF *BRASSICA OLERACEA* SUBSP. *ITALICA* AND *BRASSICA OLERACEA* SUBSP. *CAPITATA* USING TDZ AND NAA

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Abstract

Broccoli (*Brassica oleracea* subsp. *italica*) cv. Green Dragon King and cabbage (*Brassica oleracea* subsp. *capitata*) cv. Gianty are important vegetable crops grown in Cameron Highlands, Malaysia. The cotyledons of both cultivars were used as explant source for *in vitro* shoot regeneration. The objective of this research was to examine the influence of the growth regulators thidiazuron (TDZ) and α -naphthaleneacetic acid (NAA) on adventitious shoot formation in these cultivars. This system of adventitious shoot regeneration from cotyledon explants could be useful as a tool for genetic transformation of the subspecies.

Cotyledon explants of both cultivars excised from 5-day-old *in vitro* germinated seedlings were placed on shoot induction medium containing basal salts of Murashige and Skoog (MS) and various concentrations of TDZ and NAA. The highest percentage of cotyledon explant of broccoli cv. Green Dragon King producing shoot (76.66%) and the highest mean number of shoots produced per explant (0.9) were obtained on 0.1 mg/l TDZ with 0.1 mg/l NAA. Meanwhile, the highest percentage of cotyledon explant of cabbage cv. Gianty producing shoots (86.67%) and highest number of shoots produced per explant (1.1) were recorded on 0.5 mg/l TDZ with 0.1 mg/l NAA. Therefore, 0.1 mg/l TDZ with 0.1 mg/l NAA and 0.5 mg/l TDZ with 0.1 mg/l NAA are the recommended combinations for adventitious shoot regeneration from cotyledonary explants of broccoli cv. Green Dragon King and cabbage cv. Gianty respectively.

Introduction

Brassica oleracea comprises the major vegetables of the *Brassicaceae* family and almost every part of the plants are utilized including the leaves (cabbage and kale), terminal (early cauliflower) and axillary buds (Brussels sprout), flower buds (broccoli), floral primordia (late cauliflower) and stem (kohlrabi) (Katz, 2003). *Brassica oleracea* crops have many medical uses. Cabbage and broccoli are highly vulnerable to high temperature and these vegetable crops grow best in cool climate (Warland *et al.*, 2006), they are mostly cultured during the winter or early spring or on the highlands. The high temperature stress, affect both their vegetative and reproductive stages (Singh *et al.*, 2006).

The most popular method of adventitious shoot formation in *Brassica* crops which has been widely reported is through organogenesis. Other micropropagation techniques, such as somatic embryogenesis, is less applied in this genus (Cardoza & Stewart, 2003). Various tissues have been used in organogenesis of *Brassica* crops like hypocotyls (Cardoza & Stewart, 2003; Munir *et al.*, 2008), cotyledons and leaves (Kennedy *et al.*, 2005; Javed *et al.*, 2012), shoot tip (Widiyanto & Erytrina, 2001; Abbas *et al.*, 2012; Asim, 2012) thin layers of epidermal and subepidermal cells (Klimaszewska & Keller, 1985), roots (Kranthi *et al.*, 2005), and protoplasts (Kaur *et al.*, 2006). Direct organogenesis has been achieved in a variety of *Brassica* species such as from the stem sections of *Brassica juncea* (Barfield & Pua, 1991) petioles of *Brassica napus* (Ghnaya *et al.*, 2007), hypocotyls of *Brassica napus* (Phogat *et al.*, 2000) and cotyledonary explants of *Brassica campestris* ssp. *pekinensis* (Zhang *et al.*, 1998).

In vitro regeneration is influenced by many factors such as culture environment, culture medium composition,

explant source and genotype (Zhang *et al.*, 1998; Rajicic *et al.*, 2001; Bano *et al.*, 2010). The system is often used as a model for various physiological, biochemical, genetic and structural investigations in plants (Viana & Mantell, 1989). Cotyledon explant is commonly used in plant tissue culture technique to stimulate shoot initiation and the combination of BAP and NAA has been applied for shoot regeneration in many species (Guo *et al.*, 2005; Dai *et al.*, 2009; Garshasbi *et al.*, 2012). However, reports on regeneration of adventitious shoots from cotyledon explant of *Brassica oleracea* were few and faced with limited success (Teo *et al.*, 1997; Ravanfar *et al.*, 2011). Therefore, the main objective of this study was to determine the effect of TDZ either alone or in combination with NAA on *in vitro* adventitious shoot formation from cotyledon explants of broccoli cv. Green Dragon King and cabbage cv. Gianty.

Materials and Methods

Seed sterilization and germination: Hybrid seeds of broccoli cv. Green Dragon King and cabbage cv. Gianty (Leckat Corporation Sdn Bhd) were surface sterilized for 1 minute in 70% ethanol solution followed by continuous agitation for 10 minutes in 20% Clorox solution (1.05% sodium hypochlorite) with two drops of Tween 20. The seeds were rinsed four times in sterile distilled water. Seeds of broccoli were surface sterilized for 2 minutes in 80% ethanol solution followed by continuous agitation for 18 minutes in 30% Clorox solution (0.53% sodium hypochlorite) with three drops of Tween 20. Seeds were rinsed four times in sterile distilled water. The seeds of both cultivars were finally cultured on germination medium consisting of half-strength MS salts (Murashige & Skoog, 1962) with 3% sucrose and 2.8 g/l phytagel.

Explant preparation and culture: Well expanded broccoli and cabbage cotyledons from 5-day-old *in vitro* germinated seedlings were cut into halves and inoculated horizontally on the surface of medium. pH of media was adjusted to 5.7 ± 0.02 prior to autoclaving at 121°C , 1.03 kPa for 20 minutes. Explants were cultured in 100 ml flasks containing 40 ml medium and sealed with aluminum foil. The cultures were maintained at $24 \pm 2^\circ\text{C}$ and 16-h photoperiod using cool white fluorescent light providing an intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Experiment 1: Effect of TDZ and NAA on multiple shoot formation from broccoli explants (cv. Green Dragon King): The concentrations of thidiazuron (TDZ) were 0, 0.05, 0.1, 0.5 and 1 mg/l combined with α -naphthalene acetic acid (NAA) at 0, 0.1 and 0.5 mg/l. Data on percentage of explant producing shoots and mean number of shoots produced per explant were recorded after eight weeks of culture. Shoots were sub-cultured to fresh medium every four weeks.

Experiment 2: Effect of TDZ and NAA on multiple shoot formation from cabbage explants (cv. Gianty): The concentrations of TDZ were 0, 0.05, 0.1, 0.5 and 1 mg/l combined with NAA at 0, 0.1 and 0.5 mg/l. Data on percentage of explant producing shoots and mean number of shoots produced per explant were recorded after eight weeks of culture. Shoots were sub-cultured to fresh medium every four weeks.

Experimental design and statistical analysis: 30 explants for each treatment were arranged in a Completely Randomized Design (CRD) with 3 replication. Data were statistically analyzed according to Compton, 1994. Duncan New Multiple Range Test (DNMRT) at $\alpha = 5\%$ was used for comparison between treatment means. Data on mean and percentage of shoot formation on broccoli and cabbage were transformed with $\log_{10}(y + 0.1)$ and the best treatments in both experiments were analyzed by two independent sample test (T-Test) (Hara & Kotze, 2010).

Results and Discussion

Cotyledon segments of broccoli cv. Dragon King were placed on MS medium containing different concentrations and combinations of TDZ and NAA. The explants began to expand after three weeks of culture. After 8 weeks, significant differences were observed between the treatments on percentage of explants forming shoot (Fig. 1). The highest percentage of shoot formation (76.66%) was in treatment containing 0.1 mg/l TDZ and 0.1 mg/l NAA. It showed significant difference to the rest of the treatments except with 0.1 mg/l TDZ + 0.5 mg/l NAA and 0.5 mg/l TDZ. Meanwhile no significant difference in percentage of explants with shoots was observed between 0.1 mg/l TDZ and 0.5 mg/l TDZ + 0.1 mg/l NAA. NAA alone (0.1 and 0.5 mg/l) and in combination with TDZ (0.05 and 1 mg/l) also did not differ significantly from each other in terms of percentage of shoot formation.

The highest mean number of shoots per cotyledon explant (0.9) of broccoli cv. Green Dragon King was obtained in treatment containing 0.1 mg/l TDZ and 0.1 mg/l NAA (Fig. 2) which differed significantly from the other treatments. No significant difference in mean shoot number was observed between 0.1 mg/l TDZ and 0.5 mg/l TDZ +

0.1 mg/l NAA. Plate 1 shows shoot formation from cotyledon explants of broccoli cv. Green Dragon King on MS medium containing different concentration of TDZ with NAA.

We observed in cabbage explants (cv. Gianty) that the cotyledons swelled after the first week of culture and by the fourth week shoots emerged. By the 8 week of culture multiple shoots were formed. The highest percentage of shoot formation (86.67%) was observed in treatment containing 0.5 mg/l TDZ + 0.1 mg/l NAA (Fig. 3). This treatment did not show significant difference with treatments 0.05 mg/l TDZ+0.1 mg/l NAA, 0.05 mg/l TDZ+0.5 mg/l NAA, 0.1 mg/l TDZ, 0.1 mg/l TDZ+0.1 mg/l NAA, 0.1 mg/l TDZ+0.5 mg/l NAA and 0.5 mg/l TDZ, on percentage of explant with shoot. Meanwhile, media containing only NAA (0.1 and 0.5 mg/l) and in combination with 1 mg/l TDZ did not produce any shoot (Fig. 3).

TDZ at 0.5 mg/l+0.1 mg/l NAA also produced the highest mean number of shoots per cotyledon explant (1.1) followed by 0.5 mg/l TDZ (0.6) (Fig. 4). No significant difference on mean number of shoot per explant was observed among treatments incorporated with only NAA but those treatments differed significantly with the rest of the treatments except 0.05 mg/l TDZ, 1 mg/l TDZ, 1 mg/l TDZ+0.1 mg/l NAA and 1 mg/l TDZ+0.5 mg/l NAA (Fig. 4). Plate 2 shows multiple shoot formation from cotyledon explants of cabbage cv. Gianty on medium incorporated with 0.5 mg/l TDZ and 0.1 mg/l NAA after 4 and 8 weeks of culture.

Table 1 compares the best combination of TDZ and NAA on percentage of shoot formation and mean number of shoots produced per cotyledon explant of broccoli and cabbage through T-TEST. Significant difference was observed on the percentage of shoot formation between broccoli and cabbage, whereby cabbage produced higher percentage of shoot formation (86.67) than broccoli (76.66). However no significant difference was observed in terms of mean number of shoot formed per explant between the best combinations of TDZ and NAA for the two subspecies.

In this study, the wide range of TDZ concentration used, with or without NAA, influenced adventitious shoot proliferation on cotyledon explants of broccoli cv. Green Dragon King and cabbage cv. Gianty. The low concentration of 0.1 mg/l TDZ alone caused low number of adventitious shoot proliferation with or without callus formation in broccoli (Fig. 1).

The best treatment on multiple shoot formation from cotyledon explants of broccoli was 0.1 mg/l TDZ with 0.1 mg/l NAA, producing 76.66 % shoot formation and 0.9 shoots per explant respectively. TDZ (0.1- 0.5 mg/l) alone or in combination with NAA (0.1- 0.5 mg/l) stimulated adventitious shoot formation of broccoli and also enhanced shoot elongation (Fig. 1, Plate 1D). Media containing only NAA at 0.1 and 0.5 mg/l did not produce any shoots in broccoli cv. Green Dragon King (Plate 1A). The addition of 0.5 mg/l NAA into MS medium with high concentration of TDZ (1.0 mg/l) caused explant swelling instead of producing shoots (Fig. 2). There was also no shoot formation on 1.0 mg/l TDZ alone. TDZ at high concentration (1 mg/l and above) reduced shoot multiplication and TDZ at 0.1 mg/l with 0.1 mg/l NAA, besides enhancing shoot multiplication, also triggered elongation in broccoli cv. Green Dragon King.

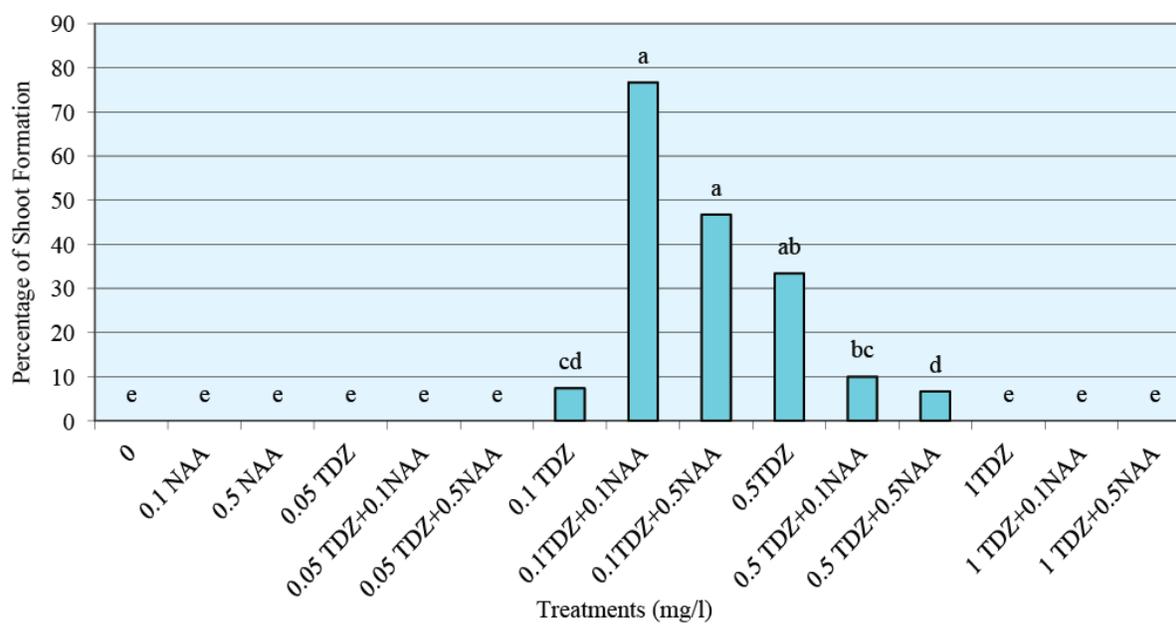


Fig. 1. Effect of different concentrations of TDZ in combination with NAA on percentage of cotyledon explants of broccoli cv. Green Dragon King producing shoots after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMR test.

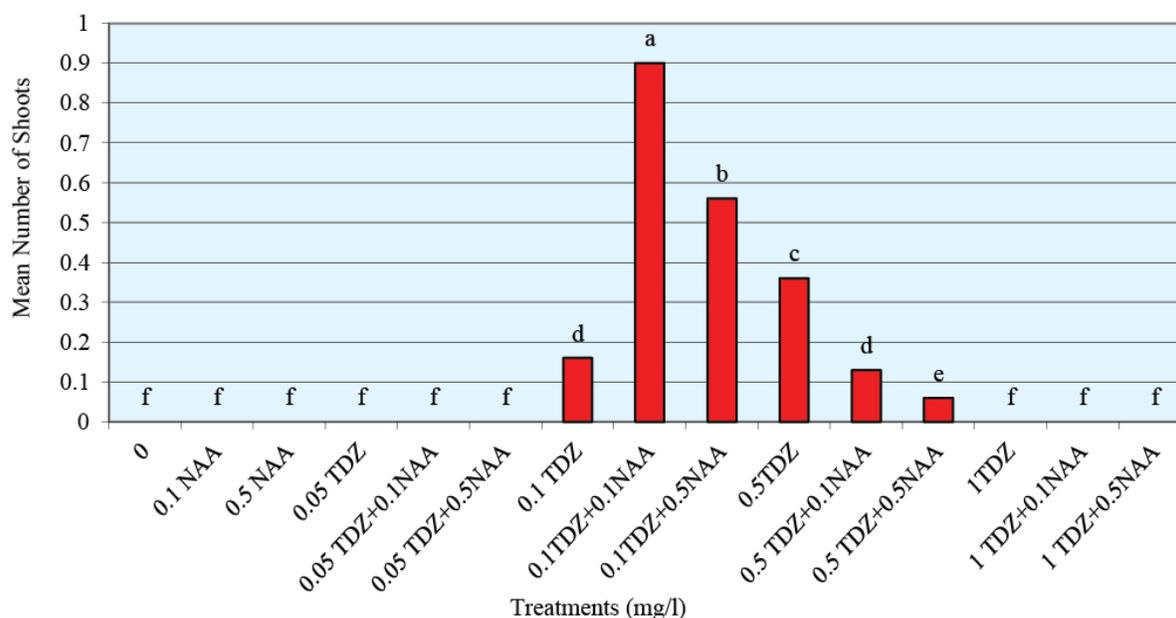


Fig. 2. Effect of different concentrations of TDZ in combination with NAA on mean number of shoots produced per cotyledon explant of broccoli cv. Green Dragon King after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMR test.

This study shows that the use of TDZ in an appropriate ratio with NAA increased shoot formation from cotyledon explants of broccoli cv. Green Dragon King. Guo *et al.*, (2005) reported that the combination of TDZ and NAA produced more shoots than BAP alone on cotyledon and leaf segment of stem mustard (*Brassica juncea* var. *tsatsai*). However, according to Ravanfar *et al.*, (2009 and 2011), BAP gave the highest number of shoots per hypocotyl and cotyledon segment with or without NAA on different cultivar of broccoli (*Brassica oleracea* subsp. *italica* cv. Green Marvel).

Varied responses could be due to genotypic differences of the cultivar or plants reacting differently during micropropagation.

In experiment 2, cotyledons of cabbage cv. Gianty cultured on 0.5 mg/l TDZ in combination with 0.1 mg/l NAA initially exhibited swelling and then differentiated to produce shoots (Fig. 3 and 4, Plate 2A). The culture of cotyledon segments on MS medium containing different concentrations of NAA alone did not produce any shoots, instead the cotyledon segments turned brown after eight weeks of culture (Figs. 3 and 4).

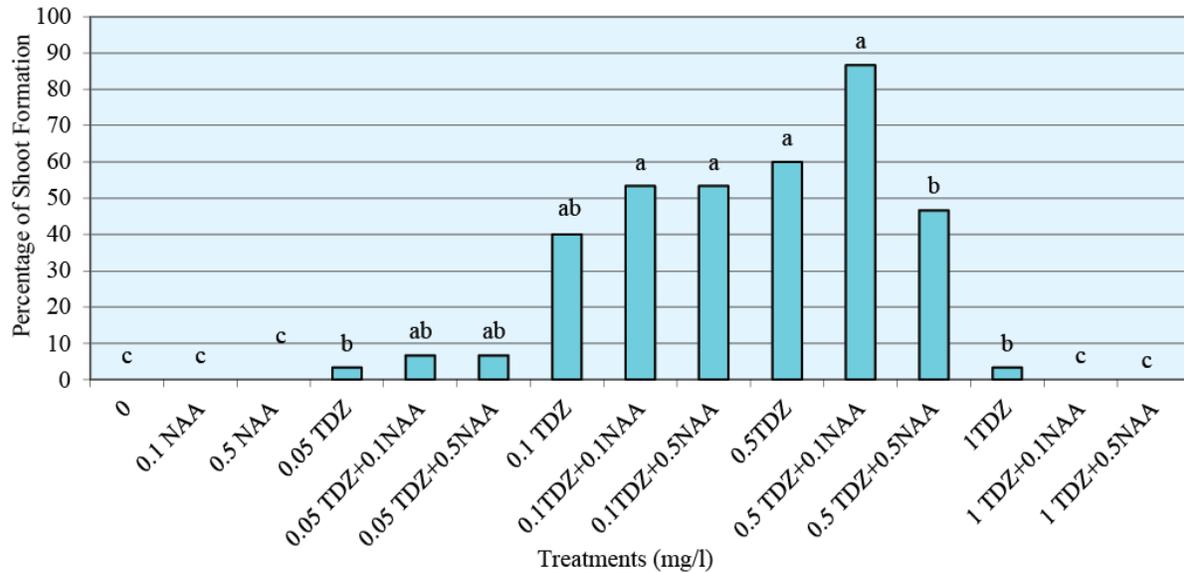


Fig. 3. Effect of different concentrations of TDZ in combination with NAA on percentage of cotyledon explants of cabbage cv. Gianty producing shoots after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

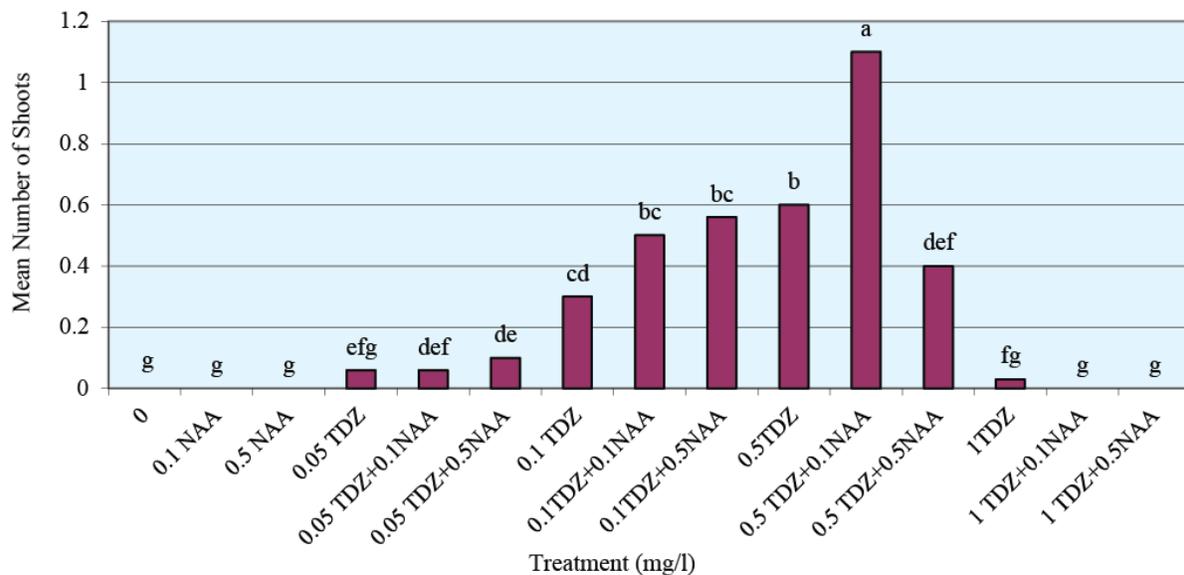


Fig. 4. Effect of different concentrations of TDZ in combination with NAA on mean number of shoots produced per cotyledon explant of cabbage cv. Gianty after eight weeks of culture. Means with the same letter were not significantly different at 0.05 probability level according to DNMRT test.

Table 1. Comparison of the best combination of TDZ with NAA on percentage of shoot formation and mean number of shoots produced per cotyledon explants of broccoli and cabbage after eight weeks of culture.

(*) significant at 0.05 level and (NS) no significant difference.

Groups	Shoot formation (%)	Sd	Se	T	p-value
Broccoli (0.1 mg/l TDZ+0.1 mg/l NAA)	76.66	5.77	3.33	-3.48	0.025*
Cabbage (0.5 mg/l TDZ+0.1 mg/l NAA)	86.67	5.77	3.33		
Groups	Mean no. of shoot per explant	Sd	Se	T	p-value
Broccoli (0.1 mg/l TDZ+0.1 mg/l NAA)	0.9	0.1	0.05	-2.43	0.072 NS
Cabbage (0.5 mg/l TDZ+0.1 mg/l NAA)	1.1	0.1	0.05		

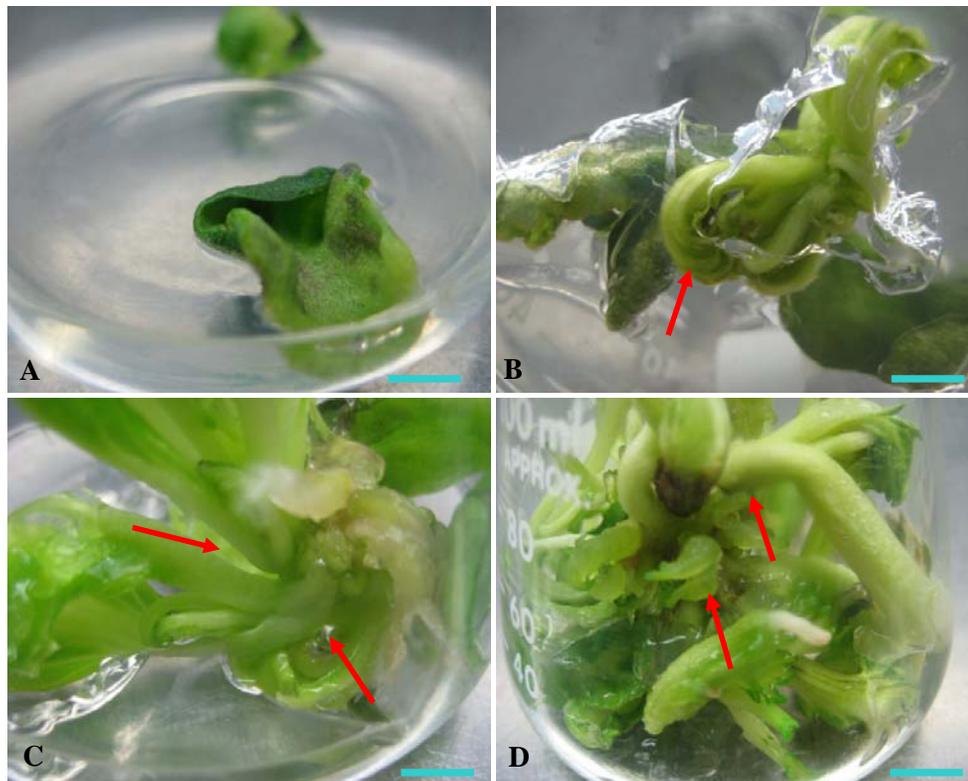


Plate 1. Multiple shoot formation from cotyledon explants of broccoli cv. Green Dragon King on different concentrations of TDZ in combination with NAA. A (0.5 NAA), B (0.1 mg/l TDZ +0.1 mg/l NAA), C (0.5 mg/l TDZ) after four weeks of culture and D (0.1 mg/l TDZ +0.1 mg/l NAA) after eight weeks of culture. Red arrow = shoot. (A, B and C) bar = 5 mm (D) bar = 7 mm.

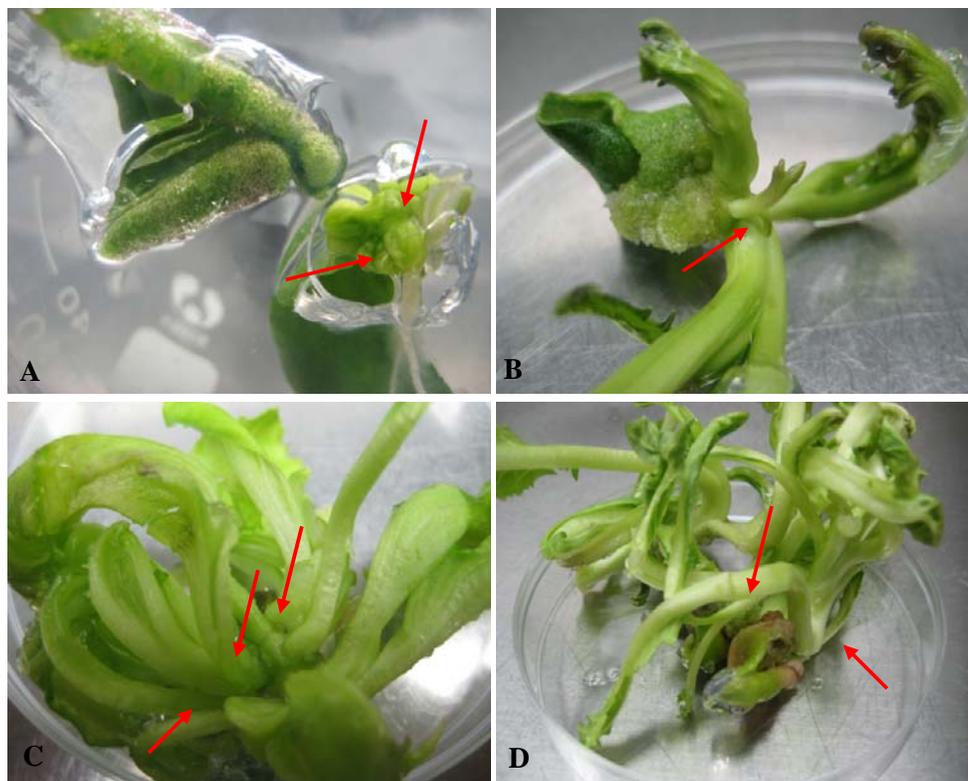


Plate 2. Multiple shoot formation from cotyledon explants of cabbage cv. Gianty on 0.5 mg/l TDZ with 0.1 mg/l NAA. (A and B) Shoot emergence after four weeks of culture. (C and D) Multiple shoot formation after eight weeks of culture. Red arrow= shoot. (A, B and C) bar = 3.5 mm (D) bar = 10 mm.

Up to a maximum of 10 shoots was attained from a single cabbage cotyledon explant after 2 successive subcultures on medium containing 0.5 mg/l TDZ 0.1 mg/l and NAA. Similarly, other researchers have reported the enhanced effect of TDZ in combination with NAA on shoot regeneration from cotyledon and hypocotyl explants of *Brassica rapa* L. ssp. *Rapifera* (turnip) (Guang *et al.*, 2008). Treatments of low concentration of TDZ alone (0.05- 0.1 mg/l) could stimulate shoot formation from the cabbage cotyledon explants, but the number of shoots produced per explant was low. The higher concentration of 1 mg/l TDZ initiated some shoots but the shoots were abnormal and there was increased callus formation. However the abnormal shoots became normal upon culturing on MS medium without any hormones for one month. Reports have indicated shoot proliferation without callus formation and abnormality on medium containing low TDZ concentration (0.3 mg/l) in *Brassica napus* (Jonoubi *et al.*, 2004) and *Brassica juncea* (Guo *et al.*, 2005). Adventitious shoot formation increased over successive culture on medium containing TDZ in combination with NAA but eventually resulted in more aberrant shoot development (Jonoubi *et al.*, 2005).

The study indicated that the ability of shoot formation from cotyledon explants of cabbage cv. Gianty was more than broccoli cv. Green Dragon King. About 86.67% of cabbage produced shoots on medium containing 0.5 mg/l TDZ and 0.1 mg/l NAA after eight weeks of culture. However the highest regeneration of broccoli was 76.66% in treatment containing lower concentration of 0.1 mg/l TDZ with 0.1 mg/l NAA.

The few or no shoot formation on media containing 0.5 mg/l NAA in both the experiments on broccoli and cabbage could be due to the auxin having a more important role in cell division and callus formation. Nevertheless, the presence of a cytokinin, which in this study was TDZ enhanced adventitious shoot formation as has been reported in often researches (Pierik, 1999; George *et al.*, 2008; Rafat *et al.*, 2010).

Conclusion

The study showed that the cotyledon explants of both the broccoli and cabbage cultivars were potential explants for *in vitro* shoot regeneration. The use of TDZ as a cytokinin and NAA as an auxin in an appropriate ratio was most essential for shoot induction and multiplication from the cotyledon explants. Experimental results also showed that cotyledon explants of cabbage (cv. Gianty) have higher ability of producing shoots than broccoli cv. Green Dragon King. The media containing only TDZ or NAA caused explant swelling and produced fewer or no shoots. TDZ at 0.1 mg/l with 0.1 mg/l NAA and TDZ at 0.5 mg/l with 0.1 mg/l NAA were the recommended combinations for shoot regeneration from cotyledon explant of broccoli cv. Green Dragon King and cabbage cv. Gianty respectively.

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