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Note

Influence of phenyl-urea and adenine-type cytokinins on direct adventitious shoot regeneration of cabbage (*Brassica oleracea* subsp. *capitata*) "KY Cross"

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Abstract In this study, the effects of phenyl-urea (Thidiazuron) and adenine (6-benzylaminopurine) and 6-y,y,dimethylally-amino purine type cytokinins alone or in combination with indole-3-butyric acid on shoot regeneration from hypocotyl and cotyledonary explants of *Brassica oleracea* ssp. *capitata* "KY Cross" were investigated. For hypocotyl explants, medium containing 2.27 μM Thidiazuron showed the highest mean number (18.15) of separable shoots per explant with 80% shoot formation. In the case of cotyledonary explants, the highest mean number of shoots (3.03) was obtained on medium containing 12.30 μM 6-y,y,dimethylally-amino purine with a percentage of 56.67% shoot formation. Plantlets were successfully acclimatized with 70% survival in potting medium consisting of coconut husk+vermicompost (7:1 v/v). The regeneration system developed herewith will be a valuable tool for genetic improvement of cabbage "KY Cross".

Key words: *Brassica oleracea* subsp. *capitata* "KY Cross", hypocotyl, shoot organogenesis, phenyl-urea cytokinin, adenine-type cytokinin.

Brassica oleracea ssp. *capitata* or commonly known as cabbage is among members of the Cruciferous family commonly associated with numerous health benefits such as containing high levels of antioxidants, anticancer compounds and vitamins A, B1, B2 and C (Christey and Braun 2001; Rafat et al. 2010; Singh et al. 2006). In Malaysia, the production of cabbage was estimated around 30 metric ton per hectare. This ranks Malaysia among the world exporter of cabbage and the Cameron Highlands is the main cultivation area for cabbage (Anem 2010).

Despite the various health benefits and nutritional content, this crop is highly vulnerable towards heat. The formation of the leafy head is greatly influenced by climate and the crop grows best in a cool climate (optimum temperature of 24°C) (Shinohara 1980) like Cameron Highlands. However, due to the land scarcity and rapid development, and recent increase in temperature in the Cameron Highlands, the production of cabbage "KY Cross" has been much affected. Highland cabbage like cultivar "KY Cross" is in greater demand than lowland cabbage due to its superior quality in terms of vigor, uniformity, disease resistance and good horticultural traits including long shelf-life and stable high yield. Malaysia imports most hybrid seeds from countries like Japan, China, Taiwan and Thailand (Anem 2011) including cultivar "KY Cross". The production of heat tolerant "KY Cross" cabbage through genetic modification is most relevant and thus the need for an efficient plant regeneration system.

There are many reports of plant regeneration through direct organogenesis (Hedayat et al. 2009; Ravanfar et al. 2011). Direct organogenesis has been achieved in a variety of *Brassica* species such as from the petioles (Ghnaya et al. 2007) and hypocotyls of *Brassica* napus (Phogat et al. 2000). Guang et al. (2008) established an efficient procedure for direct regeneration of turnip (*B. rapa* L. ssp. *rapifera*) whereby the effects of Thidiazuron (TDZ), 6-benzyladenine (BA) and naphthalene acetic acid (NAA) on adventitious shoot induction from cotyledon and hypocotyl explants were examined. High-frequency regeneration in cotyledon (90%) was achieved in combination of TDZ and NAA. Genotype specificity is a limiting factor in *Brassica* tissue culture

Abbreviations: BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid; MS, Murashige and Skoog; TDZ, thidiazuron; 2iP, 6-(y,y,dimethylally-amino)-purine; PGR, plant growth regulator.

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and regeneration, which severely limits the germplasm that can be manipulated or improved (Cardoza and Stewart 2004). In addition, there is lack of information on organogenesis of cabbage particularly "KY Cross" using phenyl-urea type cytokinin such as TDZ. Since the discovery of the first cytokinin which is kinetin in 1955, the definition of cytokinins has grown to include a large array of natural and synthetic compounds, adenine and phenylurea derivatives. Cytokinins are mostly in the form of adenine and produce two immediate effects on undifferentiated cells including the stimulation of DNA synthesis and increased cell division (Ting 1982). Cytokinins also produce a delayed response in undifferentiated tissue which is the formation of shoot primordia. TDZ is a urea derivative and does not contain the purine ring which is common to the adenine-type cytokinins such as BAP and 2iP (Lu 1993). It was suggested that TDZ was more effective than BAP due to its high cytokinin activity caused by an induced synthesis of endogeneous purine cytokinin (Thomas and Katterman 1986). The present study was undertaken to examine the effects of phenyl-urea and adenine type cytokinins on in vitro shoot regeneration from hypocotyl and cotyledonary explants of cabbage "KY Cross".

Seeds of cabbage "KY Cross" (Takii & Co. Ltd., Japan) obtained from Cameron Highland, Pahang, Malaysia were washed thoroughly for 15 min under running tap water, immersed in 70% ethanol for 2 min and rinsed once with sterile distilled water. Seeds were then treated with 20% (v/v) Clorox® (5.25% sodium hypochlorite) (Clorox (M) Pte. Ltd.) added with 1–2 drops of Tween® 20 (Merck Schuchardt, Hohenburn, Germany) for 15 min and rinsed with sterile distilled water thrice before blotting on Whatman No. 5 filter paper. Surface sterilized seeds were germinated on PGR-free half strength MS (Murashige and Skoog 1962) medium supplemented with 1.5% (w/v) sucrose and 0.45% (w/v) Gelrite™ (Duchefa, Haartem, The Netherlands). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 1.03 kPa for 20 min. Cultures were incubated at 25±2°C and 16/8-h photoperiod (light/dark) provided by white fluorescence tubes of 30 μmol m^{-2} s^{-1} light intensity.

Hypocotyl explants (5–10 mm in length) and cotyledons (cut into halves) derived from 7 day-old in vitro germinated seedlings of cabbage "KY Cross", were cultured horizontally on the surface of MS medium supplemented with 3% (w/v) sucrose, 0.45% (w/v) Gelrite™ and supplemented with three different types of PGR at various concentrations. TDZ was tested at 0.05, 0.23, 0.45, 2.27, 4.54 and 9.08 μM, BAP at 2.45, 4.90, 7.35, 9.80, 12.25 and 14.70 μM, and 2iP at 2.46, 4.92, 7.38, 9.84, 12.30 and 14.76 μM for both hypocotyl and cotyledonary explants. The control treatment was PGR-free MS medium. In addition, TDZ, BAP and 2ip in combination with IBA (Table 2) were also tested for cotyledonary explants since earlier reports (Rafat et al. 2010; Ravanfar et al. 2009) indicated poor shoot regeneration in terms of percentage and mean shoot number when cytokinins were applied alone. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 1.03 kPa for 20 min. Cultures were incubated at 25±2°C and 16/8-h photoperiod (light/dark) provided by white fluorescence tubes of 30 μmol m^{-2} s^{-1} light intensity. Subculture was carried out at four weeks interval. Parameters including the percentage of explants forming shoots, number of shoots per explant, number of leaves per explant and mean length of shoots were recorded after 8 weeks of culture. Shoots regenerated from the best treatment were transferred on PGR-free MS medium for elongation and root induction. Successfully rooted shoots, 3–4 cm in height, were transferred into potting medium containing coconut husk and vermicompost (7 : 3 v/v) and acclimatized in a misting chamber. After one month, the plants were transferred to a greenhouse under controlled environment at 25°C. They were irrigated twice a day while the anti-fungal Benozide 50WT® (Hextar Chemicals Pte. Ltd., Klang, Malaysia) and Wellgro® fertilizer were applied alternatively at 2 weeks interval.

All experiments were conducted in a Randomized Complete Block Design (RCBD) with each treatment replicated 3 times and each replication per treatment contained 10 explants. Data were analyzed using the Analysis of Variance (ANOVA) and post hoc Duncan New Multiple Range Test (DNMRT) at $p \leq 0.5$ using SAS 9.2 software. All data were subjected to One Sample Kolmogrov–Smirnov analysis to test on data normality before the analysis of variance was performed.

Morphogenic response of hypocotyl explants to shoot formation appeared within 2 weeks of incubation in all treatments. Adventitious microshoots proliferated from the middle and distal end of the explants. The results (Table 1) demonstrated that an optimum concentration of each cytokinin is crucial for successful enhancement of adventitious bud formation and shoot proliferation. Each cytokinin has different ability to induce shoots within the concentrations tested. The best percentage of hypocotyl explants forming shoots (90%) after 8 weeks of culture was obtained on MS medium supplemented with 9.84 μM 2iP, followed by 80% shoot regeneration on media supplemented with 0.23 and 2.27 μM TDZ and 77% on medium with 4.90 μM BAP.

Among all treatments, TDZ at 2.27 μM proved most effective for multiple shoot induction producing 18.15 separable shoots per hypocotyl explant (Table 1). This was followed by 0.05 and 0.23 μM TDZ producing 11.58 and 9.38 shoots, respectively. It was interesting to note that there was a strong tendency of obtaining higher shoot numbers in the presence of TDZ compared to BAP and 2iP. Supplementation of MS medium with 2iP at 9.84, 12.30 and 14.76 μM produced moderate number

Table 1. Adventitious shoot regeneration from hypocotyl explants of *Brassica oleracea* ssp. *capitata* "KY Cross" using various concentrations of TDZ, BAP and 2iP after 8 weeks of culture.

Cytokinin	Concentration (μM)	% of explant forming shoots	Mean No. of shoot±SE	Mean No. of leaves±SE	Mean length of shoot (cm)±SE
Control	0	46.67^{cd}	3.10±0.75^{efg}	1.50±0.64^{h}	3.10±1.02^{abc}
Phenyl-urea (TDZ)	0.05	33.33^{d}	11.58±1.16^{b}	5.38±0.39^{bc}	3.35±0.15^{ab}
	0.23	80.00^{ab}	9.38±1.10^{c}	5.70±0.33^{b}	3.64±0.09^{a}
	0.45	46.67^{cd}	4.75±1.01^{def}	4.15±0.26^{bcde}	3.30±0.03^{ab}
	2.27	80.00^{ab}	18.15±1.28^{a}	7.25±0.25^{a}	3.80±0.15^{a}
	4.54	36.67^{d}	5.33±0.19^{de}	8.36±0.46^{a}	2.48±0.11^{abcdef}
	9.08	53.33^{c}	3.83±0.34^{efg}	7.19±0.97^{a}	3.20±1.12^{abc}
Adenine (BAP)	2.45	40.00^{d}	2.19±0.12^{g}	2.18±0.19^{hg}	0.98±0.07^{ef}
	4.90	76.67^{abc}	2.65±0.14^{fg}	3.09±0.37^{defg}	1.38±0.19^{cdef}
	7.35	56.67^{c}	2.57±0.24^{fg}	3.23±0.50^{defg}	0.89±0.07^{ef}
	9.80	63.33^{bc}	2.68±0.34^{fg}	2.62±0.01^{efgh}	1.04±0.03^{ef}
	12.25	50.00^{c}	2.63±0.22^{fg}	2.49±0.21^{fgh}	1.21±0.12^{def}
	14.70	50.00^{c}	1.66±0.57^{g}	1.48±0.64^{h}	0.70±0.25^{f}
Adenine (2iP)	2.46	43.44^{cd}	2.15±0.75^{g}	2.42±0.65^{fgh}	1.60±0.52^{bcdef}
	4.92	70.00^{bc}	3.93±1.22^{efg}	3.85±0.07^{cdef}	3.00±1.02^{abcd}
	7.38	70.00^{bc}	3.21±0.60^{efg}	3.60±0.87^{defg}	4.10±1.80^{a}
	9.84	90.00^{a}	6.39±0.59^{d}	4.42±0.64^{bcd}	2.79±0.20^{abcde}
	12.30	73.33	6.77±0.58^{d}	4.36±0.28^{bcd}	2.24±0.12^{abcdef}
	14.76	76.67^{abc}	4.80±0.23^{def}	4.62±0.16^{bcd}	2.20±0.09^{abcdef}

Means with the same alphabet within the same column are not significantly different (DNMRT, $p \geq 0.05$). Each treatment replicated 3 times and each replication per treatment contained 10 explants.

of separable shoots at 6.39, 6.77 and 4.80, respectively. However, BAP showed the least response with the highest shoot production at only 2.68 on 9.80 μM.

TDZ at 2.27, 4.54 and 9.08 μM produced among the highest number of leaves per shoot which differed significantly from other treatments. Generally, 2iP and BAP produced lower number of leaves per shoot in comparison to TDZ. Meanwhile, both TDZ and 2iP significantly produced higher mean shoot length compared to BAP within the range of 1.60 to 4.10 cm. The application of TDZ induced varied responses on the hypocotyl explants.TDZ at 0.05 and 0.23 μM promoted up to 40% rooting of the explants by week 8 of culture. TDZ at 4.54 μM and above occasionally induced callus formation from the explant, shoot fasciation as well as explant necrosis and bleaching. Nonetheless, all shoots produced on TDZ rooted successfully after 4 weeks on PGR-free MS medium.

Morphogenic response of cotyledonary explants occurred after 3 to 5 weeks of culture for all treatments, which was comparatively slower than the hypocotyl explants. Most explants initially swelled followed by callus formation before shoots were formed. Microshoot proliferation appeared at the cut ends of the cotyledons while in some treatments roots were simultaneously induced.

In this study, TDZ showed zero response on shoot regeneration when tested alone. Interestingly, upon combining TDZ with IBA, shoot proliferation occurred (Table 2). Among the highest percentage of shoot regeneration (56.67%) was recorded on media containing

Figure 1. Morphogenetic response of cabbage "KY Cross" hypocotyl explants. A. Shoot proliferating from the distal end of a hypocotyl explant after 1 week of culture, B. high shoot frequency on 2.27 μM TDZ after 8 weeks of culture, C. adventitious roots initiated from a hypocotyl explant after 2 weeks on 0.05 μM TDZ, D. profuse rooting of shoots after 1 month on PGR-free MS medium (white bar: 2 mm, yellow bar: 1 cm).

12.25 μM BAP and 12.30 μM 2iP, respectively (Table 2). Meanwhile, the highest shoot number produced from the cotyledonary explants (3.03) was on medium containing 12.30 μM 2iP but did not differ significantly from the shoot numbers (2.83 and 2.77) obtained on the best BAP treatment (9.80 μM) and the best TDZ+IBA treatment (0.45+4.90 μM), respectively. Among the highest shoot

Table 2. Adventitious shoot regeneration from cotyledonary explants of *Brassica oleracea* ssp. *capitata* "KY Cross" using various cytokinins and their concentration of TDZ+IBA, BAP and 2iP after 8 weeks of culture.

Cytokinin	Concentration (μM)	% ofexplant forming shoots	Mean no. of shoot±SE	Mean no. of leaves±SE	Mean length of shoot (cm)±SE
Control	0.00	0.00^{g}	0.00^{d}	0.00^{h}	0.00^{g}
TDZ	0.23	0.00^{g}	0.00^{d}	0.00^{h}	0.00^{g}
	0.45	0.00^{g}	0.00^{d}	0.00^{h}	0.00^{g}
	2.27	0.00^{g}	0.00^{d}	0.00^{h}	0.00^{g}
	4.54	0.00^{g}	0.00^{d}	0.00^{h}	0.00^{g}
TDZ+IBA	0.23+4.90	53.33^{ab}	2.00±1.35^{abc}	2.25±0.65^{ab}	1.09±0.11^{abcdef}
	0.45+2.45	46.67^{bc}	1.30±0.58^{c}	1.61±0.49^{bcdefg}	0.92±0.34^{bcdef}
	0.45+4.90	43.33^{c}	2.77±0.41^{ab}	2.13±0.32^{abc}	1.02±0.20^{bcdef}
	2.27+2.45	40.00^{cd}	1.17±0.35^{c}	1.74±0.09^{abcdef}	1.10±0.13^{abcdef}
	4.54+2.45	43.33^{c}	1.30±0.58^{c}	1.33±0.58^{cdefg}	1.09±0.37^{abcdef}
BAP	2.45	33.33^{ed}	1.03±0.18^{c}	0.89±0.12^{g}	0.66±0.19^{def}
	4.90	30.00^{e}	0.87±0.35^{c}	1.03±0.29^{efg}	0.63±0.17^{def}
	7.35	46.67^{bc}	2.80±0.40^{ab}	1.93±0.01^{abcd}	1.22±0.15^{abcde}
	9.80	46.67^{bc}	2.83±0.22^{ab}	2.07±0.13^{abc}	1.41±0.05^{ab}
	12.25	56.67^{a}	1.77±0.47^{bc}	1.89±0.32^{abcd}	1.28±0.28^{abc}
	14.70	46.67^{bc}	1.17±0.78^{c}	1.11±0.56^{defg}	0.56±0.20^{ef}
BAP+IBA	2.45+2.45	20.00^{e}	0.60±0.45^{cd}	0.89±0.15^{g}	0.89±0.35^{bcdef}
	4.90+2.45	33.33^{ed}	0.87±0.38^{c}	1.00±0.29^{efg}	0.68±0.17^{def}
	7.35+2.45	40.67^{bc}	1.80±0.45^{bc}	1.80±0.35^{abcd}	0.89±0.15^{bcdef}
	9.80+2.45	46.67^{bc}	2.05±0.10^{ab}	2.17±0.13^{ab}	1.10±0.15^{abcdef}
	12.25+2.45	43.33^{c}	1.33±0.50^{c}	1.15±0.58^{cdefg}	1.09±0.30^{abcdef}
	14.70+2.45	10.00^{fg}	0.10±0.12^{d}	0.50±0.01^{gh}	0.56±0.25^{def}
2iP	4.92	26.67^{e}	0.87±0.20^{c}	0.88±0.29^{g}	0.54±0.12^{f}
	7.38	46.67^{bc}	0.93±0.48^{c}	1.10±0.47^{defg}	0.70±0.10^{cdef}
	9.84	46.67^{bc}	2.63±0.33^{ab}	1.86±0.09^{abcde}	1.35±0.14^{abc}
	12.30	56.67^{a}	3.03±0.48^{a}	2.48±0.23^{a}	1.72±0.15^{a}
	14.76	40.00^{cd}	0.80±0.25^{c}	0.97±0.17^{fg}	0.58±0.03^{ef}
2iP+IBA	4.92+2.45	36.67^{d}	0.80±0.38^{c}	0.81±0.33^{g}	0.68±0.17^{def}
	7.38+2.45	40.67^{cd}	0.87±0.45^{c}	0.80±0.35^{abcd}	0.89±0.35^{bcdef}
	9.84+2.45	40.67^{cd}	1.03±0.25^{c}	0.88±0.23^{g}	0.90±0.15^{bcdef}
	12.30+2.45	43.33^{bc}	1.33±0.50^{c}	0.90±0.58^{cdefg}	1.09±0.30^{abcdef}
	14.76+2.45	16.37^{f}	0.10±0.35^{d}	0.30±0.01^{h}	0.50±0.01^{ef}

Means with the same alphabet within the same column are not significantly different (DNMRT, $p \geq 0.05$). Each treatment replicated 3 times and each replication per treatment contained 10 explants.

length attained was 1.72 cm on medium with 12.30 μM 2iP after 8 weeks of culture. The rest of the cotyledonary explants only swelled and formed callus without any shoot regeneration throughout the 8 weeks of culture.

Plantlets, 3–6 cm in height and with well-developed root system (consisting of primary and secondary roots), derived from the hypocotyl and cotyledonary explants were removed from the culture flasks and transferred to pots (5 cm in diameter) containing coconut husk+vermicompost (7 : 1 v/v). The media had been previously autoclaved at 121°C for 20 min. The rooted shoots were acclimatized under control environment in a misting chamber. A survival rate of 70% was achieved a month after the transplantation. The plants were then transferred to a greenhouse with an average temperature of 23°C for compact cabbage head formation. Compact cabbage heads began to form after a month in the greenhouse and ready for harvest 2 months later.

The influence of plant growth regulators especially cytokinins on growth and development of in vitro cultured tissues is critical in order to maximize plant regeneration. Pierik (1999) mentioned that in vitro culture is often impossible without the addition of plant growth regulators. TDZ, a phenyl-urea cytokinin has been widely reported to exhibit higher biological activity compared to the most active adenine type cytokinins such as BAP and kinetin (Debnath 2006; Mok and Mok 2001; Munshi et al. 2007). In this study, high number of shoot formation (18.15) was obtained with TDZ at its optimal concentration of 2.27 μM from hypocotyl explants of cabbage "KY Cross". This finding was in parallel to previous studies reported on apples (*Malus* sp.) and hackberry (*Celtis occidentalis* L.) (Garelkova and Alexieva 1992; Marinova and Iliev 1992). Though the highest percentage of hypocotyl explants forming shoots (90%) after 8 weeks of culture was on MS medium supplement with 9.84 μM 2iP it did not differ significantly with that attained on 0.23 and 2.27 μM TDZ (80%). This result suggested that the percentage of hypocotyl explants that responded to form shoots was

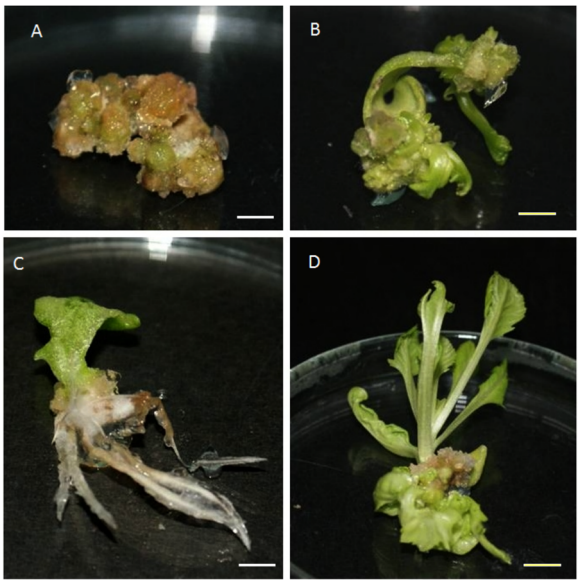

Figure 2. Morphogenetic responses of cabbage "KY Cross" cotyledonary explants. A. Callus covering the cotyledonary explant on medium with only TDZ after 8 weeks of culture, B. adventitious shoot produced from the cut end of a cotyledonary explant on medium with 9.80 μM BAP after 4 weeks of culture, C. medium containing 0.23 μM TDZ+4.90 μM BAP initiated adventitious root formation after 5 weeks of culture, D. shoot growth after 8 weeks of culture on medium with 12.25 μM BAP (white bar=0.5 cm, yellow bar=1.0 cm).

cytokinin type and dose dependent. High frequency of shoot regeneration was reported from hypocotyl explants of *Brassica alboglabra* using different concentrations of 2iP (Pua et al. 1989)

It was also noticed in this study that there are differences in the mode of action of the different cytokinin types in term of shoot proliferation and shoot morphological appearance. TDZ displayed more noticeable defects especially above its suboptimal concentration (4.54 μM) where the shoots produced were slightly bleached, appeared glassy and exhibited sign of hyperhydricity. Meanwhile, callus occasionally appeared asynchronously on the hypocotyl explants that were exposed to 4.54 μM TDZ and above but was absent in all treatments containing adenine-type cytokinins. The occurrence of callusing on media incorporated with TDZ had been reported in other studies (Faisal and Anis 2005; Guo et al. 2005; Sharma and Shahzad 2008; Stevens and Pijut 2012). Increasing the TDZ concentration to 4.54 μM and above significantly suppressed the regeneration capacity of the hypocotyl explants. According to Lu (1993) TDZ has the potential of inducing endogenous auxin synthesis in both hypocotyl and cotyledonary explants. The assumption of TDZ-mediated modulation of endogenous auxins is evidently seen in this study on cabbage "KY Cross" whereby adventitious roots and shoots were concurrently induced from the hypocotyl explants at low concentrations of 0.23 and 0.05 μM TDZ.

Interestingly in this study, BAP was less effective than both TDZ and 2iP on the hypocotyl explants, which is in opposition to the findings of Kadota et al. (2001), and Kadota and Nimi (2003) who found BAP to have more noticeable effect than TDZ on shoot multiplication of pear. It was also in contradiction with Sedlák and Paprštein (2007) who found 2iP unsuitable for shoot proliferation in sweet cherry. In this study, 2iP at the suboptimal concentration (12.30 μM) could produce 6.77±0.58 shoots per explant, while at 9.84 μM exhibited the highest percentage of explant forming shoots.

In the case of cotyledonary explants, no shoot regeneration was obtained in this study when TDZ was utilized alone, but showed synergistic response when combined with IBA. Cheng et al. (2001) and Guo et al. (2005) reported on the enhanced effect of TDZ when combined with NAA on shoot regeneration from cotyledon explants of *Brassica oleracea*. Meanwhile, the adenine-type cytokinins (BAP and 2iP) generally acted better when applied alone instead of in combination with IBA, particularly with respect to the percentage of shoot formation and mean number of shoots as shown in Table 2. Although there were significant differences among the treatments in terms of shoot number, leaf number and shoot length produced by the cotyledonary explants, the phenyl-urea cytokinin TDZ in combination with IBA did not show any better response than the adenine-type cytokinins (BAP and 2iP) tested alone or in combination with IBA on shoot regeneration from cotyledonary explants of cabbage "KY Cross".

Nonetheless, this study demonstrated that the ability of regeneration from cotyledonary explants of cabbage "KY Cross" was not in consonance with earlier reports in other *Brassica* (Chi et al.1991; Dunwell et al.1981; George and Rao, 1980) in which cotyledonary explants showed better regeneration potential than hypocotyl explants.

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