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Full Length Research Paper

Effect of high concentration of thidiazuron (TDZ) combined with 1H-indole-3-butanoic acid (IBA) on Albion strawberry (*Fragaria* × *ananassa*) cultivar plantlets induction

Rudi Hari Murti[1], Samir C. Debnath[2] and Young Rog Yeoung[1]*

[1]Department of Plant Sciences, Gangneung-Wonju National University, Gangneung 210-702, Korea.
[2]Atlantic Cool Climate Crop Research Centre, Agriculture and Agri-Food Canada, 308 Brookfield Road, Bldg. 25, St. John's, NL A1E 5Y7, Canada.

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This study was conducted to understand the effects of high concentration of thidiazuron (TDZ) combined with 1H-indole-3-butanoic acid (IBA) on production of strawberry plantlets. Young folded leaves of "Albion", "Seolhyang" and "Akihime" cultivars were collected, dipped in a detergent solution and then sterilized by treating with 70% (v/v) ethanol. The leaves were cut into 2 to 3 mm diameter pieces. Different concentrations of TDZ combined with IBA were supplemented in Murashige and Skoog (MS) medium to induce direct organogenesis. Plantlet regeneration from leaf explants was detectable at 3 weeks after culture. Plantlet regeneration rate of "Albion" was more than 60% but was very poor for 'Akihime' and 'Soulhyang' cultivars. The effect of TDZ and IBA concentrations and their interactions were significant for plantlet regeneration rate and plantlet number per explant. Plantlet regeneration rate increased with increment of TDZ concentrations up to 40.9 µM and achieved regeneration rate of 96% and 11.3 plantlets per explant and the addition of IBA (0.5 or 2.5 µM) improved regeneration rate up to >70%. The optimum concentration of TDZ was 37.8, 39.2 and 40.5 µM when combined with 2.5, 0.5 µM IBA and without IBA, respectively. It took 16 weeks from leave culture to complete plantlets. As a result, a protocol for "Albion" plantlet regeneration on MS medium containing high TDZ concentrations combined with IBA was developed.

Key words: Thidiazuron, 1H-indole-3-butanoic acid, organogenesis, regeneration rate, plantlets, *Fragaria* × *ananassa*.

INTRODUCTION

Adventitious plantlet regeneration has been demonstrated in a variety of strawberry explants including leaves (Passey et al., 2003; Yonghua et al., 2005; Debnath, 2005, Folta et al., 2006; Landi and Mezzetti, 2006; Husaini and Abdin, 2007;Mohamed et al., 2007), petioles (Passey et al., 2003; Debnath, 2005, 2006, 2009; Folta et al., 2006), stipules, roots (Passey et al., 2003), sepals (Debnath, 2005, 2006, 2009), *in vitro*-produced bud (Jemmali et al., 2002) and stolons (Folta et al., 2006). Of these, leaf tissue has been the most studied and shown to result in high regeneration frequencies and plantlet number per explant (Passey et al., 2003; Husaini and Abdin, 2007; Mohamed et al., 2007). The plantlet regeneration response of leaf explants has mainly been related to the genotype, plant growth regulator (PGR)

*Corresponding author. E-mail: yryeoung@gwnu.ac.kr.

Abbreviations: TDZ, Thidiazuron; **IBA,** 1H-indole-3-butanoic acid; **PGR,** plant growth regulator.

type, concentration and the type of nutrient medium employed in the protocol.

Thidiazuron (TDZ), a substituted phenylurea with cytokinin- and auxin-like effects, is regarded as a highly efficacious bio-regulator for morphogenesis in the tissue culture of many plant species (Murthy et al., 1998). TDZ alone (Debnath, 2005) or in combination with (2,4-dichlorophenoxy) acetic acid (2,4-D) (Passey et al., 2003) or IBA (Hanhineva et al., 2005; Yonghua et al., 2005), was found to be effective for plantlet regeneration from strawberry leaves. Researchers applied TDZ between 0.5 (Debnath, 2005) and 18 µM (Husaini and Abdin, 2007) in regenerating explants and the effect of TDZ depended on the concentration and genotype. Husaini and Abdin (2007) found that 9 µM TDZ stimulated direct regeneration via *de novo* shoot bud formation in leaf disk and 18 µM induced direct embryogenesis. Rapid propa-gation and high stress levels triggered by hormone concentration (Sansavini et al., 1990) and the route of somatic embryogenesis (direct or via a callus stage) (Biswas, 2009) resulted in high somaclonal variation due to epigenetic events (Kaeppler et al., 2000) or from variation pre-existing in the mother plant (James et al., 2007).

In strawberry, the TDZ effect has been identified in some cultivars and the response depends on the genotype. “Albion” strawberry with its high productivity is a potential genotype for genetic manipulation. Information on micropropagation of “Albion” strawberry is not available. In the present study, we established an *in vitro* plantlet regeneration protocol of this cultivar on a nutrient medium containing IBA and high concentration of TDZ. The effect of IBA on improving regeneration rate and plantlet number/explant was discussed.

MATERIALS AND METHODS

The current study was conducted at the Plant Physiology Laboratory, Department of Plant Science, Gangneung-Wonju National University, South Korea on *Fragaria × ananassa* “Albion”, “Seolhyang” and “Akihime” strawberry cultivars. All genotypes were grown in 800 m above sea level with hydroponic system using polyvinyl chloride (PVC) gutters (20 cm wide, 30 cm high) suspended 1 m above green house floor. The water-nutrient supply was automatic, every hour or every thirty minutes depending on the light intensity. Complete nutrient solution was supplied through drip irrigation system with emitters at 30 cm spacing and a flow capacity of 2 L min^{-1} per 30 m tube length, containing (in mg L^{-1}): KNO_3 (286), $Ca(NO_3)2•4H_2O$ (354), $MgSO_4•7H_2O$ (123), KH_2PO_4 (91), NH_4NO_3 (7), H_3BO_3 (3), $MnSO_4•4H_2O$ (2), $CuSO_4•5H_2O$ (0.05), $ZnSO_4•7H_2O$ (0.22), $NaMoO_4•5H_2O$ (0.02) and EDTA-Fe (20). The pH and EC of the solution were 5.6 and 1.0 dS m^{-1}, respectively.

Medium and culture condition

Young folded leaves collected from the glasshouse were dipped in a detergent solution for 2 to 3 min, washed in tap water for 5 min and then sterilized by treating with 70% (v/v) ethanol for 1min followed by a 0.1% (w/v) $HgCl_2$ solution containing a drop of Tween®20 (Fluka, Germany) for 10 min. The leaves were then rinsed three times in sterile distilled water, placed on sterile-paper saturated with sterile water, to prevent desiccation, and cut into 2 to 3 mm diameter pieces. The explants were cultured on Murashige and Skoog salts and vitamins formulation (Duchefa, Harlem, The Netherlands) supplemented with 3% (w/v) sucrose (Aldrich®), 0.8% (w/v) plant agar (Duchefa, Harlem, Netherlands) and combinations of TDZ (31.8, 34.1, 36.4, 38.6, 40.9, 43.2, 45.5 and 47.7 µM) and IBA (0, 0.5, and 2.5 µM) concentrations. The factorial experiment was arranged in completely randomized design with three replications. The medium pH was adjusted to 5.7 and treatments were added to the medium before autoclaving at 121°C for 15 min. Each experimental unit consisted of three glass jars (∅ 8 cm), each containing 30 ml of medium. Twenty explants were placed in each jar with abaxial side down of the medium.

Explants were cultured in the dark for 2 weeks (Husaini and Abdin, 2007) followed by 16/8 h day/night photoperiod under 27 $\mu E/m^2/s$ (provided by fluorescent lamp, 36 W) at 23 ± 2°C. After four weeks, regenerated plantlets were subcultured with a reduced density by half per jar on the same medium. After two months, plantlets were transferred to the same medium but with 2.7 µM 6-benzylaminopurine (BA) instead of TDZ for plantlet proliferation. Proliferation phase required 4 weeks.

For rooting, elongated shoots were separated individually and cultured for 6 weeks on the same medium but without plant growth regulators. Rooted plantlets were rinsed free of substrate and rooting characters of four random plantlets from each treatment were measured. The plantlets were then planted in plug trays containing 1 peat moss : 1 vermiculite (v/v), which were kept in transparent closed humid chamber and acclimatized by gradually ambient humidity, with spraying fungicide regularly to prevent fungal attack. After 15 days, plantlets were moved to the green-house and maintained for one month and exposed under net shading (40%) for 7 days before fully exposed to sun light.

Data collection and statistical analysis

For plantlet regeneration, the frequency of explants producing bud was recorded after 8 weeks of culture, while plantlet number was observed at the 18th week. Data on primary roots and plantlet characters of four random samples were determined for each treatment. Shoots and roots were separated by cutting the roots from the bottom of the shoot and then both of them were dried in an oven at 80°C for 4 days. Fresh and dry weights (g) of shoot and root of four random plantlets were recorded using an analytical balance (sensitivity 10^{-4} g).

Data were subjected to analysis of variance (ANOVA) (95% significant level) using SAS statistic software package version 9.2 (SAS Institute, Cary, NC, USA) and means' separation were determined with Duncan’s multiple range test at $\alpha = 5\%$. Data transformation with logarithm of explants regeneration was done for stabilizing variance before analysis and back transformed to display in the tables.

RESULTS AND DISCUSSION

In “Albion” explants, regeneration was observed 3 weeks after culture but longer time was required for “Seolhyang” and “Akihime”. Plantlet regeneration from leaf explants was generated through direct organogenesis (Figure 1A and B) and detectable at 3 weeks after culture, as were also reported previously by Debnath (2006) and Husaini and Abdin (2007) on a medium containing 2 to 4 and 9

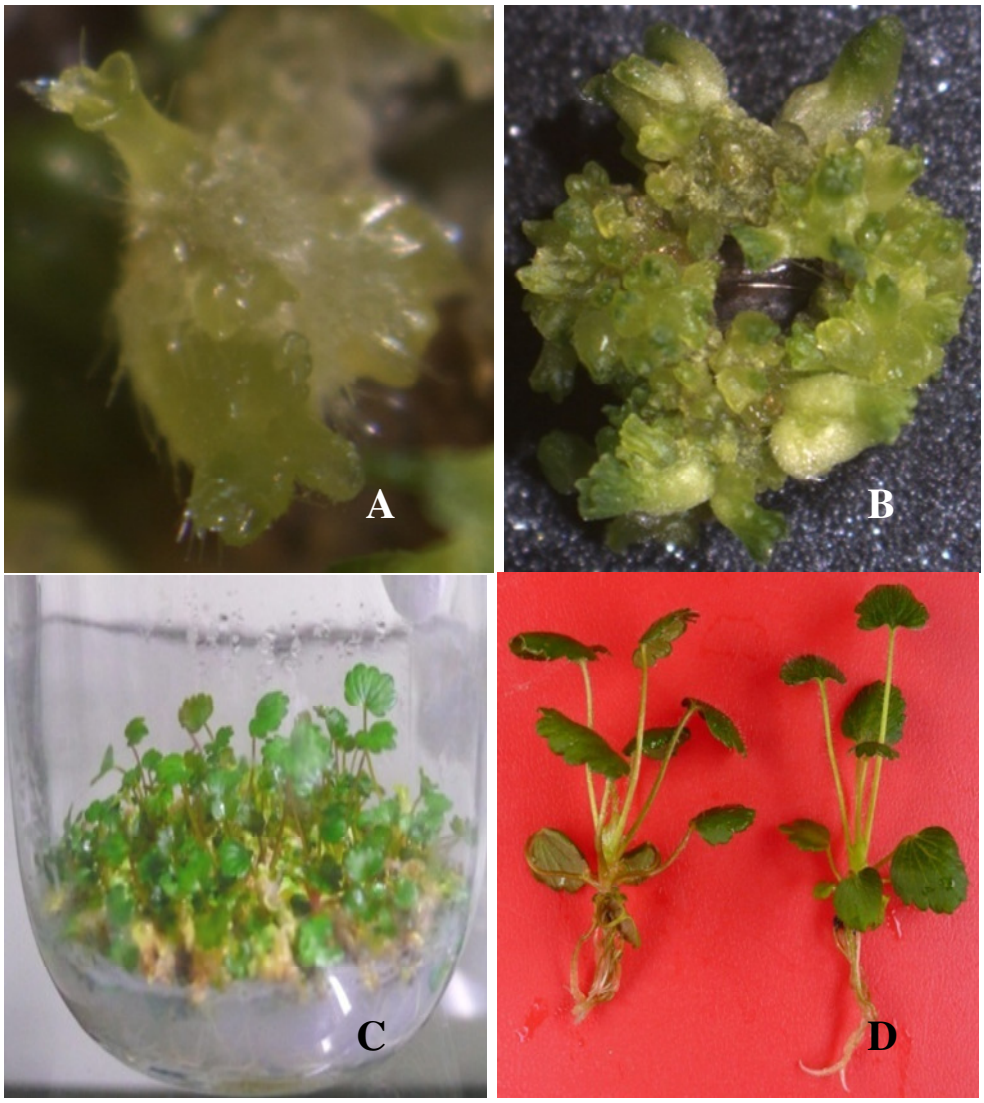

Figure 1. Stages of *in vitro* morphogenesis of "Albion" from leaf explants; A, shoot initiation after 5 weeks on basal MS medium plus 40.9 µM TDZ and 2.5 µM IBA; B, bud and shoot cluster after 6 weeks on MS + 40.9 µM TDZ and 2.5 µM IBA; C, shoot clusters after 4 weeks on MS + 2.7 µM BA; D, rooting of 12 week-old adventitious shoots (developed on MS + 2.7 µM BA) 6 weeks after transfer to MS without PGR.

µM of TDZ, respectively. Higher concentration of TDZ resulted in shoot fascination (Debnath, 2006) and somatic embryogenesis (Husaini and Abdin, 2007), while in this experiment, shoots were produced through organogenesis. It implies that the response of each genotype was different for high concentration of TDZ. Adventitious shoots appeared to be generated directly from the explants, starting on the edge cut of the leaf explants as noticed by Jalali et al. (2010). Bud were also produced from the mid and around other veins. They were noticeable on the explants after 4 to 5 weeks in culture medium after which they developed into plantlets (Figure 1D).

Plantlet regeneration rate of "Albion" using greenhouse-derived leaf explant in this experiment was more than 60% but was very poor for cultivars "Akihime" and "Soulhyang" (data not shown). Consequently, no further investigations were undertaken with the latter. The similar response for *in vitro* plantlet regeneration potential by these two cultivars in high TDZ-containing medium might be due to their genetic closeness. "Seolhyang" is the progeny between "Akihime" and "Redpearl" (Kim et al., 2009). Similar results were also reported by Passey et al. (2003) in Elsanta and Eros strawberries. Difficulty in plantlet regeneration of both cultivars was allegedly linked with inhibition by phenolic compounds (Nehra et al., 1989; Passey et al., 2003).

Analysis of variance indicated that TDZ and IBA

Table 1. Mean values for TDZ and IBA concentrations on shoot regeneration and shoot number/explant of "Albion" leaf explants after 8 weeks of culture.

Plant growth regulator	Concentration (µM)	Regeneration frequency (%)	Shoot number/ explant
TDZ	31.8	80[cz]	9.6[bc]
	34.1	65[d]	7.7[d]
	36.4	80[c]	9.0[bc]
	38.6	83[c]	9.8[b]
	40.9	96[a]	11.3[a]
	43.2	92[ab]	8.7[bcd]
	45.4	85[c]	8.5[cd]
	47.7	67[d]	1.9[e]
IBA	0	72[c]	4.1[c]
	0.5	82[b]	6.2[b]
	2.5	89[a]	15.1[a]
TDZ x IBA		*	*

Means of each PGR were pooled data; [z]Means separation was performed by Duncan multiple range test at P = 0.05; * = TDZ-IBA interaction was significant at α = 5%.

concentrations and their interactions were significantly different for the plantlet regeneration rate and plantlet number per explant (Table 1). With the tested IBA concentrations, plantlet regeneration frequency increased with increasing TDZ concentrations from 31.9 to 40.9 µM with a maximum of 96% regeneration and 11.3 plantlets per explant (Table 1). Comparable results for plantlet number were also reported by Mohamed et al. (2007). The lowest regeneration rate was on MS medium with 34.1 µM TDZ without IBA (Figure 2) which was comparable with the results of Landi and Mezzetti (2006) who used 20.4 µM TDZ + 0.98 µM IBA. The effect of TDZ without IBA on regeneration rate of explant achieved 72% with 4.1 plantlets/explant, in spite of this it was lower than result in medium enriched with TDZ and IBA. TDZ alone has also been used for strawberry shoot regeneration (Debnath, 2005, 2006, 2009), indicating that TDZ has the unique property of mimicking both auxin and cytokinin effect on growth and differentiation of cultured explant (Murthy et al., 1998).

High regeneration rate (>70%) was observed when TDZ was combined with IBA (Table 1), indicating that IBA played a crucial role in strawberry plantlet regeneration (Hanhineva et al., 2005). Figures 2 and 3 show the effects of TDZ concentrations at each IBA concentration and different responses were observed. Addition of TDZ to the medium up to 40.9 µM resulted in significantly better conditions for plantlet regeneration in medium without IBA. At 2.5 µM of IBA, TDZ concentration did not affect plantlet regeneration and plantlet number per explant. However, regeneration rate was at a very high level at all TDZ concentrations when 0.5 or 2.5 µM IBA was added in the medium (Figure 2). The estimate of optimum concentration of TDZ for regeneration rate was 39.6 and 42.5 µM when 0.5 µM IBA and without IBA was applied, respectively.

Table 1 shows that plantlet number per explants was more than 7, at all TDZ concentrations except at 47.7 µM. Highest plantlet number/explant, about 11.3 plantlets per explant, was observed at 40.9 µM TDZ, which was significantly different from other TDZ concentrations. Across IBA concentrations, 47.7 µM TDZ produced the lower number of plantlets per explant, but with more vigorous plantlets (data not shown) than other TDZ treatments. IBA had significant impact on the number of adventitious plantlets per explant as previously noted by Husaini and Abdin (2007). In all TDZ concentrations, 2.5 µM IBA produced the highest number of plantlets per explant, followed by 0.5 µM IBA and without IBA treatment (Figure 3). Plantlet number increased to 40.9 µM when TDZ without IBA was applied, then decreased with higher TDZ concentrations. A similar trend occurred in TDZ + 0.5 IBA with lower optimum concentration of TDZ than TDZ without IBA. Plantlet number per explant was more than double when 2.5 µM IBA was added to regenerating medium containing TDZ (Figure 3). These results were comparable to those of Husaini and Abdin (2007) with "Chandler" strawberry. This result indicated that the presence of auxin-like compounds in combination with cytokinin is essential for the onset of strawberry plantlets *in vitro*. The optimum concentration of TDZ (indicated by vertical lines) was estimated as 37.8, 39.2 and 40.5 µM when combined with 2.5, 0.5 µM and without IBA, respectively.

IBA and activated charcoal have been included in *in vitro* root initiation medium of strawberry (Jemmali et al.,

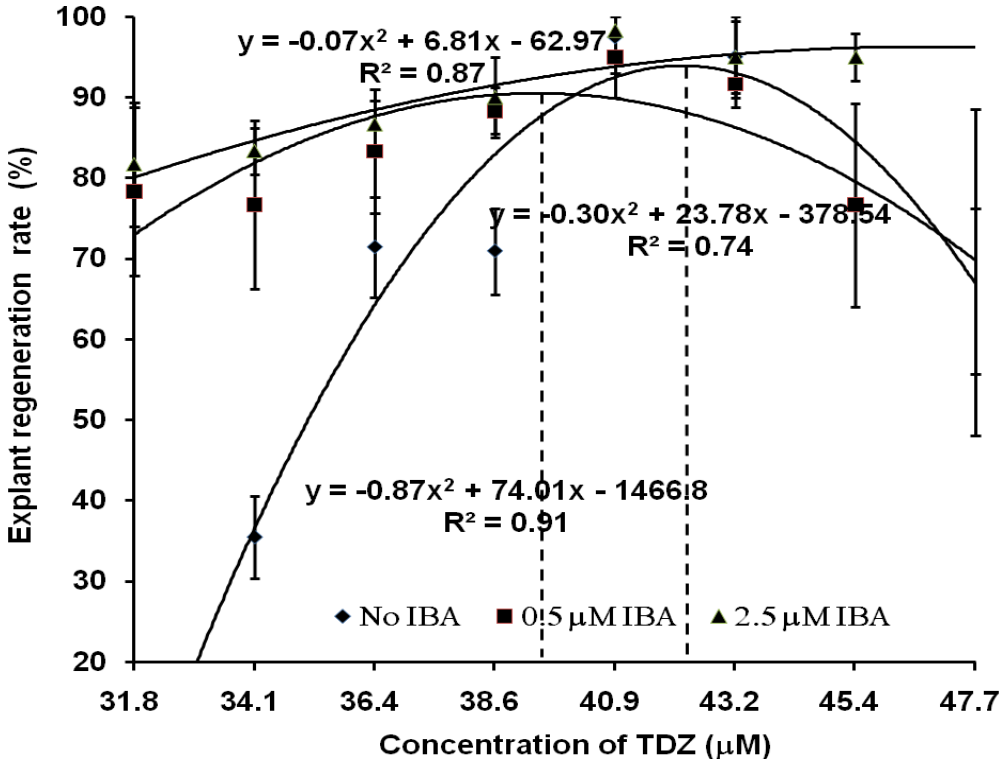

Figure 2. The effect of TDZ concentrations on regeneration rate of "Albion" strawberry leaf explants at different IBA concentrations, after 8 weeks of culture on MS medium. Vertical lines are optimum concentrations of TDZ.

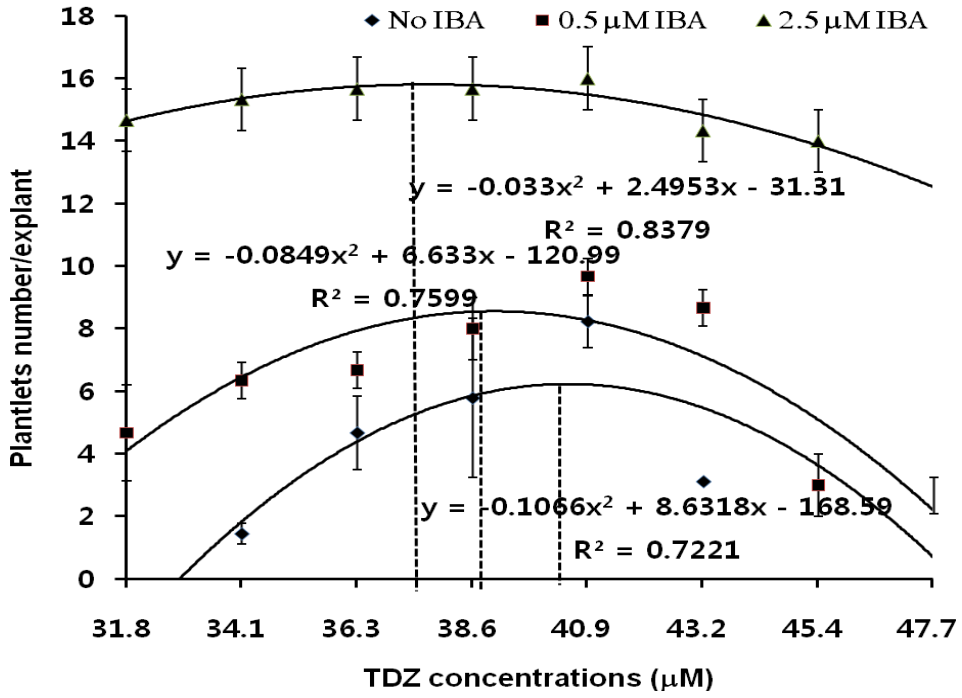

Figure 3. The effect of TDZ concentrations on plantlets number per explant of "Albion" leaf explants at different IBA concentrations after 18 weeks of culture on MS medium. Vertical lines are optimum concentrations of TDZ.

Table 2. Mean values of shoot and root parameters for plantlets produced at different TDZ and IBA concentrations.

Plant growth regulator	Concentration (µM)	Root number	Root length (cm)	Fresh weight of root (g)	Dry weight of root (g)	Leaf number	Plant height (cm)	Fresh weight of shoot (g)	Dry weight of shoot (g)
TDZ	31.8	9.0^{zd}	3.3	0.20^{c}	0.02	5.7^{d}	4.4^{cd}	0.9^{d}	0.14^{e}
	34.1	10.6^{bcd}	2.9	0.22^{c}	0.03	7.0^{c}	4.0^{d}	1.1^{cd}	0.17^{de}
	36.4	11.1^{bc}	3.1	0.25^{bc}	0.04	7.8^{ab}	4.4^{cd}	1.3^{bcd}	0.21^{cd}
	38.6	12.2^{ab}	3.1	0.34^{a}	0.04	8.3^{a}	5.0^{bc}	1.6^{ab}	0.29^{ab}
	40.9	13.3^{a}	3.0	0.36^{a}	0.04	8.2^{a}	5.5^{b}	1.6^{ab}	0.28^{abc}
	43.2	12.1^{ab}	3.0	0.31^{ab}	0.03	7.3^{bc}	5.4^{b}	1.4^{bc}	0.23^{bcd}
	45.5	10.4^{bcd}	3.1	0.33^{ab}	0.04	6.9^{c}	6.8^{a}	1.7^{ab}	0.29^{ab}
	47.7	9.8^{cd}	3.2	0.32^{ab}	0.04	7.6^{abc}	4.9^{bc}	1.9^{a}	0.32^{a}
IBA	0	11.3	2.9^{b}	0.17^{c}	0.01^{b}	9.4^{a}	3.3^{b}	1.1^{b}	0.18^{c}
	0.5	11.1	3.2^{a}	0.37^{a}	0.06^{a}	5.7^{c}	5.8^{a}	1.8^{a}	0.30^{a}
	2.5	11.4	3.0^{ab}	0.31^{b}	0.04^{a}	7.7^{b}	5.5^{a}	1.2^{b}	0.23^{b}
TDZ x IBA		*	*	*	*	*	*	ns	*

Means of each PGR were pooled data; ^{z} consisted of four plantlets from each experimental unit; the number in column followed by different letter are significantly different by DMRT α = 5%; * and ns = interaction significant and not significant, respectively.

2002). In the current study, following the study of Debnath (2005, 2006, 2009), root initiation was carried out on MS medium containing no PGR, where plantlets rooted well. The non requirement for an auxin at the rooting stage indicates that the plantlets might contain enough auxin that accumulated from the previous culture medium. Rooted plantlets (Figure 1D) were eventually acclimatized.

Variance analysis for plantlet characters showed that there were significant interactions between TDZ and IBA for all characters, except for the fresh weight of shoot (Table 2). The effects of the interaction between TDZ and IBA on plantlet morphological parameters was probably related to accumulation of the PGR and their effects during the previous culture stages. The ratio of IBA and TDZ concentration seems to be an important factor influencing the performance of plantlets. One disadvantage of TDZ is that it often inhibits shoot elongation (Cao and Hammerschlag, 2000). So, the presence of IBA in TDZ-enriched medium improved significantly both in shoot initiation and rooting.

The possible carryover effects of TDZ and IBA concentrations were significant for all characters, except for root number per plant, root length and dry weight of roots (Table 2). Root number per plant was affected by TDZ but not by IBA concentrations. The effect of IBA concentration on dry root weight was significant. The supply of IBA increased the root length, root fresh weight, plant height and shoot fresh weight. TDZ at 40.87 µM produced the highest root fresh weight and resulted in the highest number of root per plant. Increased TDZ concentrations increased leaf number, plant height and shoot fresh weight.

No consistent differences for IBA effects were detected between shoot characters in *in vitro*-derived plantlets. At 0.5 µM, the effect of IBA was significantly stronger than the other tested concentrations on almost of the plantlet characters. The number of leaves of plantlets produced in medium with TDZ but without IBA was significant higher than those produced on TDZ and IBA. Regardless of IBA concentrations, plantlets in 40.9 µM TDZ produced the best rooting system with vigorous shoot characters. Plantlets produced in other combination also had root systems suitable for stability in acclimation. Hence, the survival rate of acclimatized plants in all treatments

was almost 100%. The adequate roots of the plantlets under the transparent plastic cover during the initial 2 weeks *ex-vitro* period were the critical points for success in the acclimation of plantlets. These results are in accordance with that of Seon-Woo et al. (2010).

In the present study, for the first time, a protocol for plantlet regeneration on MS medium containing high TDZ concentrations combined with IBA was developed in "Albion", a strawberry variety that has vigorous growth and high berry yield in Korea. Based on the TDZ trend effect illustrated in the reported equation ($y = -0.07x^2 + 6.81x - 62.97$), there is an opportunity to apply even higher concentrations of TDZ than those tested here. Such conditions may promote PGR-induced somaclonal variation, which may be explored in future for the induction and selection of variants with potentially-useful traits.

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