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Scalp Induction Rate Responses to Cytokinins on Proliferating Shoot-Tips of Banana Cultivars (*Musa* spp.)

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Abstract: Problem statement: The effect of cytokinins on scalp induction from *in vitro* propagating shoot tips of different banana (*Musa* spp. AAA and AAB) cultivars was studied. **Approach:** Proliferation rate increased with an increasing concentration of Benzyl Amino Purine (BAP) up to 22.2 µM while, lower level of BAP (11.1 µM) increased scalp induction. **Results:** Kinetin induced a lower number of shoots than that obtained with similar levels of BAP and caused an increase in scalp induction rate at higher concentration (22.2 µM). Shoot proliferation in 'Rastali'(AAB), 'Berangan Intan' and 'Berangan'(AAA) significantly increased with increasing Thidiazuron (TDZ) concentration from 3.17, 2.17 and 3.33 shoots at 0.5 µM to 14.17, 6.22 and 6.17 shoots at 5 µM respectively, as concentrations above 5 µM for all cultivars were inhibitor. **Conclusion/Recommendations:** The highest ratio of scalp formation (8.89) was recorded in 'Rastali' at the highest concentration of TDZ (7.5 µM), but in 'Berangan Intan' and 'Berangan' (AAA) TDZ increased scalp induction rate from 0.00 and 0.43 at 0.5 µM to 4.22 and 2.67 at 5 µM respectively before falling to 2.11 for both at 7.5 µM. BAP at 22.2 µM was considered optimal for shoot proliferation as well as shoot elongation from excised scalps of banana cultivars.

Key words: Banana, cytokinins, proliferation rate, scalp induction, shoots tip

INTRODUCTION

The banana belonging to the genus *Musa* of the family Musaceae, is the fourth most important global food crop after rice, wheat and maize in the sense of gross value of production with a global annual production of about 88.47 million tons (Kotecha and Desai, 1995). The use of tissue culture techniques for vegetative propagation of bananas is an ordinary practice in many commercial nurseries. Shoot tip culture is the basic technique for *Musa* propagation (Ma and Shii, 1974; Swamy *et al*., 1983; Cronauer and Krikorian, 1984a; 1984b; Singh *et al*., 2004; Pua, 2007). The rate of shoot proliferation is the most important factor of micropropagation (Pua, 2007; Bairu *et al*., 2008). Plants regenerated from shoot tip culture have been shown to perform identically well or even better than those from conventional vegetative propagation under field conditions (Vuylsteke and Ortiz, 1996; Pua, 2007). Scalp which has been reported by many researchers is consisted of several fleshy bulbous structures producing tiny white tissues and looks like cauliflower (Dhed'a *et al*., 1991; Villalobos and Garcia, 2008; Sholi *et al*., 2009) possessing a highly proliferating ability as a good source for embryogenesis and providing embryogenic cell suspensions (Dhed'a *et al*., 1991; Strosse *et al*., 2004). Dhed'a *et al*. (1991) expressed that the scalp is the resultant of converted shoot tips into compact clumps of meristematic buds. The establishment of embryogenic cell suspensions from scalps suggests a better choice for plant regeneration as it can be obtained at any growth stage and thus saves the time (Sadik *et al*., 2007). Schoofs *et al*. (1998) developed a widely applicable methodology for the initiation of embryogenic cell suspensions using scalps. However, their method used a high dose (100 µM) of BAP. The protracted use of a high dose of BAP in this study had the disadvantage of

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producing somaclonal variation and also an undesirable decrease in somatic embryogenesis (Schoofs *et al*., 1998). Sadik *et al*. (2007) cultured shoots of banana cultivars on a multiplication media modified by adding a combination of BAP and TDZ for scalp generation. In their study the mean multiple bud proliferation increased as the concentration of TDZ increased in combination with BAP, they stated that higher multiple bud proliferation, which indicates better scalp formation was achieved in the treatments with TDZ than BAP.

The objective of this study was to investigate the effects of cytokinins for scalp induction in proliferating shoot tips and to study the efficiency of multiplication in scalps derived from shoot tips of banana cultivars under different concentrations of BAP. These scalps with high proliferating capacity can be used for mass clonal propagation which consequently may be the preferred target material for induced mutations and genetic engineering.

MATERIALS AND METHODS

Micropropagated cultures of banana cultivars; 'Berangan Intan', 'Berangan' (AAA group) and 'Rastali' (AAB group) were used as the source of materials for the establishment of *in vitro* shoot tip cultures. The shoot apices were trimmed to a size of approximately 5-7 mm by removing several sheathing leaves and excision with minimum basal corm tissues. Explants were inoculated in 100 mL capacity conical flasks consisting of 30 mL MS basal salts and vitamins (Murashige and Skoog, 1962) and sucrose (30 g L^{-1}), solidified with 2.8 g L^{-1} gelrite supplemented with different concentrations of Benzylaminopurine (BAP) and Kinetin (Kin) at (0.0, 11.1, 22.2, 33.3 and 44.4 µM). When designing micropropagation experiments it is often necessary to have a lower concentration range for Thidiazuron (TDZ) than the other cytokinins (Huetteman and Preece, 1993), therefore, the concentrations of TDZ were prepared at (0.0, 0.5, 2, 5 and 7.5 µM). Before autoclaving at 121°C for 20 min, the pH of the culture medium was adjusted to 5.6. Shoot tip cultures were maintained at 27±2°C under continuous light of 1500 lux. Each treatment was replicated three times with each replicate having three explants. Two months after inoculation, scalp induction at the basal end of shoot tips was observed. At the end of culture cycle, the proliferation rate responses (determined by counting all shoots outgrowths per explants) were recorded. The scalp induction rate was also recorded by counting all fleshy bulbous structures observed at basal end of proliferating shoot tips/total explants. Scalps were selected, cut back and transferred to fresh MS medium (Fig. 2B) supplemented with BAP at (0.0, 11.1, 22.2, 33.3 and 44.4 µM). After one month, the number of shoots per explants (determined by counting all shoots/scalp) and average shoot length (determined by measuring three randomly selected shoots) in response to different concentrations of BAP were recorded. The experiments were arranged in a completely randomized design with three replicates and the data collected were analyzed using SAS and MSTATC computer program and comparison of means were tested for significance, using LSD test, at 0.05 level of probability.

RESULTS

Two months after inoculation, scalp induction from proliferating shoot tips was observed (Fig. 1). These high proliferating tissues were located mostly at the basal end of developing shoot tips (Fig. 1C, 2C, D and E). The results of analysis of variance of the scalp induction response among the three cultivars showed that the scalp induction rate responses were significantly influenced by the cytokinin type, concentrations of cytokinins, banana cultivars and their interactions (Table 1). In the case of BAP treatments, there were significant differences in the rate of scalp induction and shoot multiplication under each treatment. The number of shoots significantly increased in all three cultivars with an increasing concentration of BAP up to 22.2µM (Fig. 3). Although BAP at 22.2 µM was considered optimal treatment for shoot multiplication in all banana cultivars (Fig. 3), with lower level of BAP (11.1 µM) most explants grew into a single shoot (Fig. 2C and D) or showed minimal shoot multiplication with increasing the formation of scalp at their basal parts (Fig. 2). Frequency of these *in vitro* responses to BAP were not the same among three cultivars, in the way that 'Rastali' (AAB) showed a higher multiplication and scalp formation rate than the other cultivars (AAA) (Fig. 3 and 4).

Table 1: Analysis of variance of scalp induction rates of cultivars; 'Brangan Intan', 'Berangan' (AAA group) and 'Rastali' (AAB group) on MS medium supplemented with different concentrations of cytokinins[a]

Source of variation	Df	MS	F-values
Cultivar (C)	2	79.170	3025.66**
Cytokinin (K)	2	39.400	1505.95**
Cytokinin rate (H)	4	29.930	1143.76**
C*K	4	4.510	172.52**
C*H	8	5.520	211.09**
K*H	8	25.690	981.82**
C*K*H	16	8.390	320.60**
Error	90	0.026	

*: Significant at p = 0.001; [a]: Coefficient of variation = 9.60

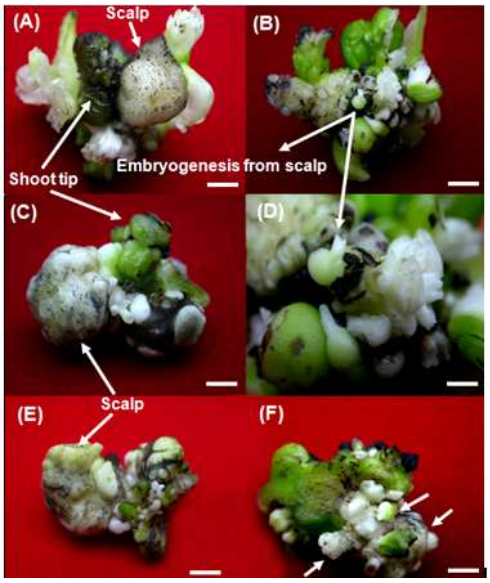

Fig. 1: Scalp induction and proliferation in micro-propagating shoot tips of banana cultivars with different cytokinins. (A) scalp formation in shoot tip of cultivar 'Berangan' on MS medium supplemented with TDZ at 2 µM. the bar scale represents 5 mm. (B) and (D) scalp formation in shoot tip of 'Berangan Intan' developing on MS medium amended with TDZ at 5 µM. Arrows show direct embryogenesis from scalp. The bar scale represent 5 and 2 mm for (B) and (D) respectively. (C) scalp formation (arrows show cauliflower like-structures) at basal end of shoot tip in 'Rastali' on MS medium supplemented with BAP at 11.1 µM. Bar scale = 5 mm. (E) and (F) scalp formation from excised shoot tips of 'Rastali' on MS medium supplemented with 7.5 µM TDZ. Arrows show scalp tissues. Bar = 5 mm

BAP at higher concentrations (33.3 and 44.4 µM) resulted in reducing of scalp induction rate (Fig. 4) and most of explants grew into a cluster of shoots. Our results showed that low concentrations of BAP (11.1 µM) also induced higher amounts of scalp at the basal end of developing shoots (Fig. 1C, 2C and 2D) than that obtained with the higher concentrations tested in this investigation (Fig. 4) which consequently offered lower concentrations of BAP as an effective treatment to induce scalp. Results indicated that increasing kinetin above 22.2 µM did not significantly increase shoot proliferation in all banana cultivars and there was no significant difference in multiplication rate between 22.2 and 33.3 µM kinetin (Fig. 5). It was concluded that kinetin induced a lower number of shoots than that obtained with similar levels of BAP.

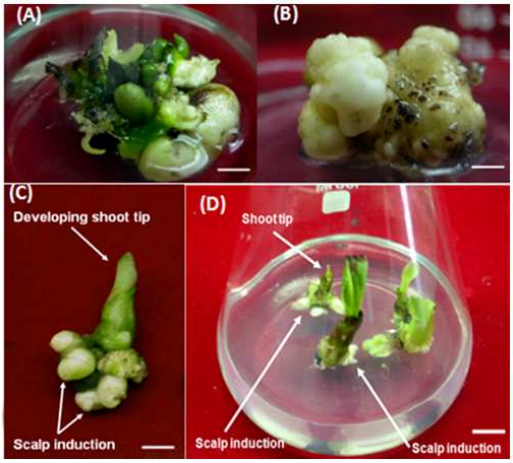

Fig. 2: (A) Scalp induced in proliferating shoot tips of 'Rastali' (AAB) on MS medium supplemented with TDZ at 5 µM. Bar = 5 mm. (B) scalp of cultivar 'Rastali' after subculture on MS medium supplemented with 11.1 µM BAP. Bar = 5 mm. (C) and (D) scalp induction and proliferation in shoot tip of 'Rastali' on MS medium supplemented with BAP at 11.1 µM. Bar = 5 mm and 10 mm in Fig. 2C and D respectively

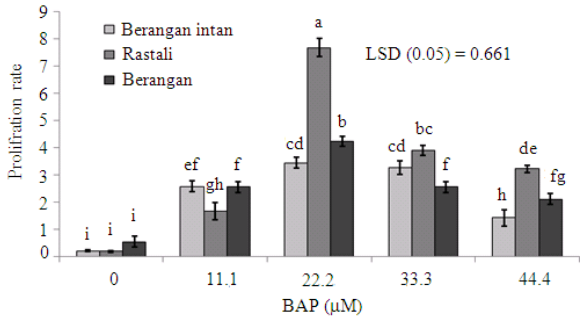

Fig. 3: Proliferation rates caused by different concentrations of BAP in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

A significant increment in scalp induction rate occurred with increasing concentrations of kinetin up to 22.2 µM (Fig. 6), which can be concluded that kinetin at higher concentration (22.2 µM) caused an increase in scalp induction rate compared to BAP(11.1 µM) which has never been reported before. In the case of 'Rastali' a similar trend was observed concerning higher ability of scalp formation at all concentrations of kinetin like BAP (Fig. 4 and 6).

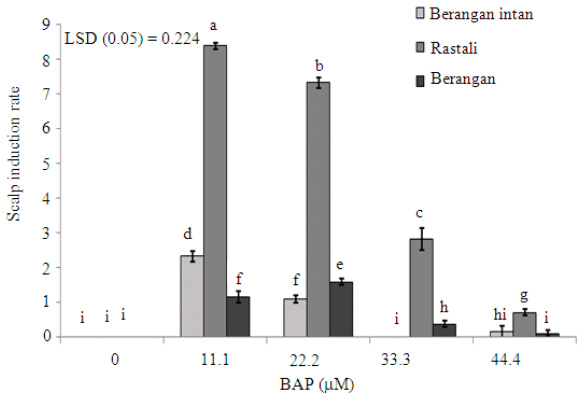

Fig. 4: Scalp induction rate responses to different concentration of BAP in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

Data obtained showed that scalp produced by kinetin treatments was small compared to BAP treatments for all cultivars (Fig. 4 and 6). The weaker activity of kinetin compared to BAP towards induction of multiple shoots was reflected in the lower average number of shoots per explants (Fig. 3 and 5). Therefore, kinetin at 22.2 µM was considered optimal for scalp induction and shoot multiplication in all banana cultivars (Fig. 5 and 6). While BAP at 11.1 µM was considered optimal for scalp induction and at 22.2 µM was suitable for shoot multiplication (Fig. 3 and 4). Similar to BAP, kinetin at very high concentrations (44.4 µM) also caused a reduction in multiplication rate as well as scalp induction (Fig. 5 and 6), but this reduction was at lower extent than that obtained with similar levels of BAP treatment. Although these *in vitro* responses to BAP and kinetin were the same among three cultivars, but results indicated that frequency of scalp induction and multiplication rate in response to BAP and kinetin was cultivar dependent, in a way that 'Rastali' (AAB group) showed higher ability of scalp induction potential in all concentrations of BAP, than 'Berangan Intan' and 'Berangan' (AAA group) (Fig. 4). The inhibitory effects of BAP on both scalp induction and multiplication rate was observed at higher concentrations (33.3 and 44.4 µM) (Fig. 3 and 4). It was clearly evident from Fig. 7 that shoot proliferation in 'Rastali' (AAB) 'Berangan Intan' and 'Berangan' (AAA) significantly increased with increasing TDZ concentration from 3.17, 2.17 and 3.33 shoots at 0.5 µM to 14.17, 6.22 and 6.17 shoots at 5 µM respectively. Higher gross of multiplication rate for all cultivars was carried out with TDZ at 5 µM and higher concentrations were inhibitor (Fig. 7).

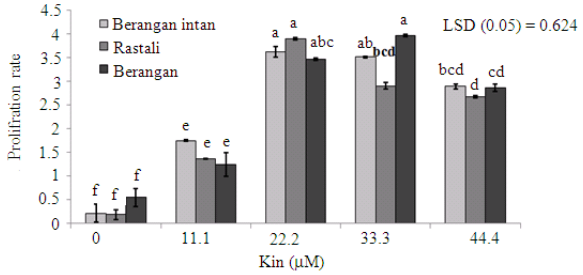

Fig. 5: Proliferation rates caused by different concentrations of kinetin in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

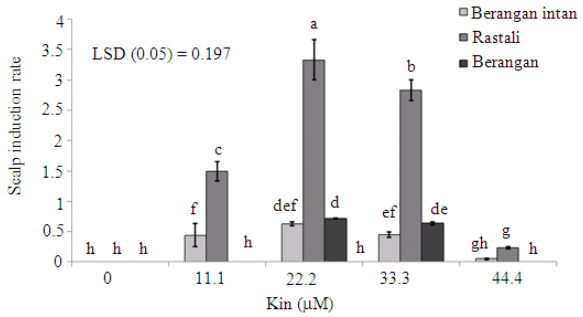

Fig. 6: Scalp induction rate responses to different concentration of kinetin in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

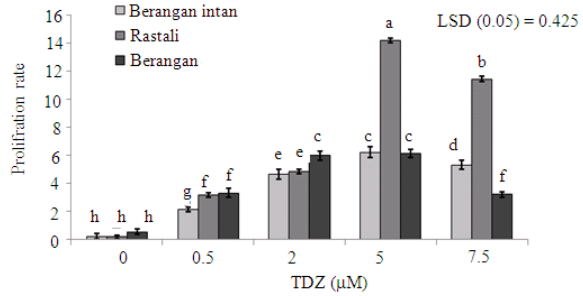

Fig. 7: Proliferation rates caused by different concentrations of TDZ in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

In the case of 'Rastali'(AAB) with increasing of TDZ concentration scalp induction rate was increased, as the highest gross of scalp formation rate (8.89) was recorded at the highest concentration of TDZ (7.5 µM),

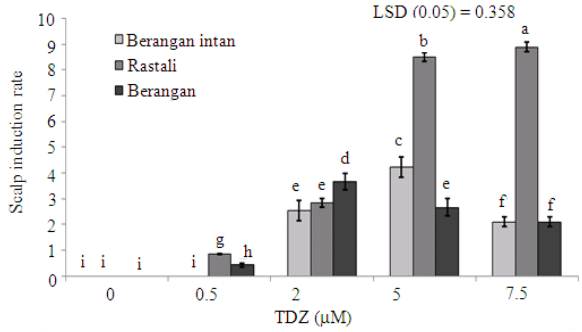

Fig. 8: Scalp induction rate responses to different concentration of TDZ in shoot tips of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

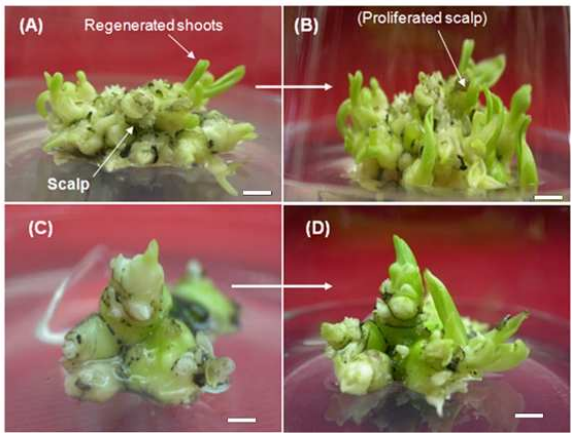

Fig. 9: (A) and (C) scalp of 'Rastali' (AAB) and 'Berangan Intan'(AAA) 10 days after transferring on MS supplemented with BAP at 22.2 µM respectively, (B) and (D) proliferated scalp of 'Rastali' and 'Berangan Intan' 20 days after transferring on MS supplemented with BAP at 22.2 µM, respectively. Bar = 5 mm

but in 'Berangan Intan' and ' Berangan' (AAA) TDZ increased scalp induction rate from 0.00 and 0.43 at 0.5µM to 4.22 and 2.67 at 5 µM respectively before falling to 2.11 for both at 7.5 µM, as the highest concentration of TDZ was inhibitor for these cultivars (AAA), (Fig. 8).

An interesting observation was considerable affects of TDZ on ability of scalps for embryogenesis as most scalps were triggered the formation of embryo immediately after induced with TDZ (Fig. 1B and D), it may therefore, be worthwhile to investigate the comparative effect of TDZ and other cytokinins for induction of embryogenic scalp.

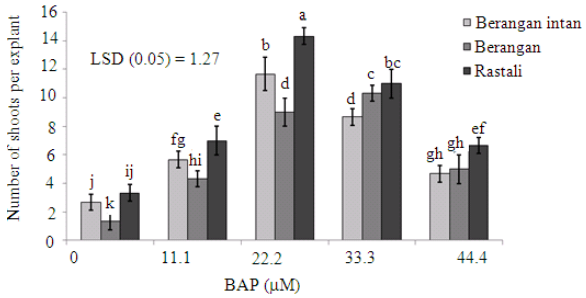

Fig. 10: The effect of different concentration of BAP on average number of shoots per scalp in banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

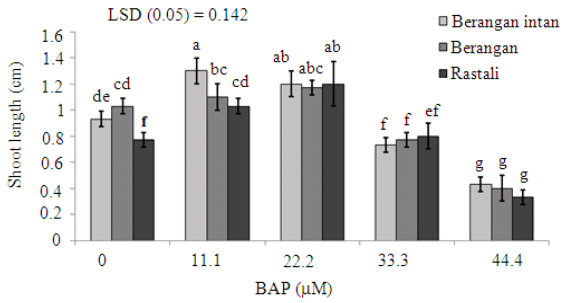

Fig. 11: The effect of different concentration of BAP on average shoots length in scalp of banana cultivars. Different letters indicate values are significantly different at the 0.05 probability level according the LSD test

Shoot regeneration from excised scalps was observed 10 and 20 days after subculture (Fig. 9 A, B, C and D). Measurable changes containing of average number of shoots/explants and shoot elongation was counted after 4th week. The data recorded indicated that proliferation rate and shoot length was increased with increasing of BAP up to 22.2 µM while BAP above 22.2 and 33.3 µM decreased the shoot length and proliferation rate in scalps (Fig. 10 and 11).

DISCUSSION

Observation of scalp as a white fleshy bulbous structure (cauliflower like-structure) has been also reported by several researchers (Dhed'a *et al*., 1991; Strosse *et al*., 2006; Sadik *et al*., 2007; Sholi *et al*., 2009). Sadik *et al.* (2007) and Sholi *et al.* (2009) stated that the formation of scalps from the multiple buds was cultivar and medium dependent which is generally in agreement with the results of this study. Venkatachalam *et al.*

(2007) showed that the number of shoots increased with an increasing concentration of BAP up to 22.2 µM, they stated that with higher levels of BAP (33–44.4 µM) a reduction in number of shoots regenerated occurred which is in agreement with the results obtained in the present study. Previous reports (Cronauer and Krikorian, 1984b; Jarret *et al*., 1985; Gubbuk and Pekmezci, 2004; Bairu *et al*., 2008) indicated that 22.2µM of BAP is the optimum treatment for most banana tissue cultures. Also there are many reports based on inhibitory effects of high concentrations of BAP on shoot multiplication rate (Gubbuk and Pekmezci, 2004; Haq and Dahot, 2007; Bairu *et al*., 2008; Strosse *et al*., 2008). Reviewing the literature on scalp induction in banana cultivars (Dhed'a *et al*., 1991; Schoofs *et al*., 1998; Sholi *et al*., 2009), we noticed that scalp induction has been reported on a broad range of BAP-enriched culture media as Dhed'a *et al*. (1991) reported that 10 µM of BAP was essential to induce scalp in plantain cultivar 'Bluggoe' (ABB) which was closer to the findings reported in this study, While, Schoofs *et al*. (1998) used a high dose of BAP (100 µM) to induce scalp. Sholi *et al*. (2009) stated that BAP at 44.4 µM was optimal for scalp induction. These differences in sensitivity of the scalp induction may also be due to cultivar dependent responses to the different BAP concentrations. Therefore, we concluded that scalp induction in banana shows a wide range of BAP dose dependent responses among cultivars and within genomic groups which is generally in agreement with Sholi *et al*. (2009) who suggested that different *Musa* genotypes and cultivars require different levels of plant growth regulator (BAP) to induce scalp formation. All these reports inferred that scalp induction responses were the resultants of two-way interactions of BAP concentration and cultivar. Regarding that banana has been proved to be a highly recalcitrant material for *in vitro* plant regeneration (Strosse *et al*., 2006; Venkatachalam *et al*., 2006) and our results create a hypothesis based on activating genes that regulate regeneration of shoot and scalp induction in bananas, It may be concluded that B genome has high scalp induction and proliferation rate potential rather than A genome, thus banana cultivar can be genetically manipulated for improving shoot regeneration and scalp production. There are several reports based on the high cytokinin activity of TDZ (Huetteman and Preece, 1993; Arinaitwe *et al*., 2000). Gubbuk and Pekmezci (2004) stated that the shoot proliferation response to TDZ were stronger than to BAP in all banana types examined in their research. Therefore, the concentration rates of TDZ were reduced are presented in Fig. 7 and 8. There are few reports about using of TDZ in *Musa* spp. Arinaitwe *et al*. (2000) were the first to study the effect of TDZ on multiplication rate in shoot tips of banana cultivars. They stated that the optimum concentration of TDZ varied significantly by cultivar. In a way that shoot multiplication in 'Ndiziwemiti' (ABB) increased with increasing TDZ concentrations, but in 'Bwara' and 'Kibuzi' (AAA) decreased with increasing concentrations, which is similar to the results found in this investigation, showing more inhibitory effects of TDZ at high concentrations on 'Berangan Intan' and 'Berangan' (AAA) than 'Rastali' (AAB) (Fig. 7). Sadik *et al*. (2007) showed that in a mixture of BAP and TDZ, low concentrations of 12.4 and 4.55 µM BAP and TDZ, respectively, were able to induce scalp formation. The results obtained in our studies showed that in contrast to BAP being the most effective at its lowest concentration (11.1µM), TDZ should be applied above 2 µM, specially for 'Rastali' (AAB) which is in agreement with those of both Dhed'a *et al*. (1991) and Sadik *et al*. (2007).

CONCLUSION

Frequency of scalp induction in banana cultivars was influenced by cultivar and the cytokinin type as in contrast to BAP being the most effective at its lowest concentration, TDZ should be applied at higher concentrations especially for 'Rastali' (AAB). Kinetin at 22.2 µM was considered optimal for scalp induction and shoot multiplication in 'Berangan Intan' (AAA), 'Berangan' (AAA) and 'Rastali' (AAB), while BAP at 11.1 µM was optimal for scalp induction and at 22.2 µM was suitable for shoot multiplication. Also BAP at 22.2 µM was considered optimal treatment for shoot proliferation as well as shoot elongation from excised scalps of banana cultivars (Fig. 10 and 11). These scalps with high proliferating capacity are suitable as a mass clonal propagation which subsequently can be the preferred target material for induced mutations and genetic engineering.

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