

Influence of salicylic acid on morphological and physiological responses of banana (*Musa acuminata* cv. 'Berangan', AAA) shoot tips to *in vitro* water stress induced by polyethylene glycol

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Abstract

Growth and productivity of banana is seriously restricted by water deficit. Salicylic acid (SA) induces biotic and abiotic stress tolerance in crops. To study the ameliorative effects of SA on water stress in banana (*Musa acuminata* cv. 'Berangan', AAA), shoot tip explants with 8 mm in size were treated with varying SA concentrations (0, 1, 2 and 3 mM) and incubated on MS media containing different levels (0, 1, 2 and 3 %) of PEG *in vitro*. After 2 months, proliferation rate, fresh weight increase, relative water content, chlorophyll level, proline accumulation, malondialdehyde (MDA) and H₂O₂ contents were measured and analyzed. The results indicated that with increasing levels of PEG, proliferation rate, fresh weight increase, relative water content and chlorophyll concentrations were significantly decreased. The SA concentrations improved shoot tips performance by increasing proliferation rate, fresh weight increase and relative water content. Although non SA – treated shoot tips were not significantly responsive to increasing levels of PEG in terms of elevated proline content, they responded positively to supply of SA by showing significant increase in proline and chlorophyll contents under water stressed conditions. SA treatments also enhanced plant tolerance against oxidative stress. This was observed through significant reduction in H₂O₂ and MDA contents of SA – treated shoot tips under water stress conditions. The results revealed that exogenous application of SA helped to reduce the harmful effects of water deficit on banana regenerants *in vitro*.

Keywords: Banana; Lipid peroxidation; Oxidative stress; Salicylic acid; Shoot tip culture; Water stress.

Abbreviations: BAP- benzyl aminopurine; DW- dry weights; FW- fresh weight; LSD- least significant difference; LWL- leaf water loss; MDA- malondialdehyde; PEG- polyethylene glycol; ROS- reactive oxygen species; RWC- relative water contents; SA- salicylic acid; TBA- thiobarbituric acid; TCA- trichloroacetic acid; TW- turgid weights.

Introduction

Drought is one of the most important factors limiting plant growth, and is claimed to reduce production on 25% of arable land throughout the World (Levitt, 1980; Farooq et al., 2009). Drought slows growth, induces stomatal closure, and therefore reduces photosynthesis (Nemeth *et al.*, 2002). Several lines of evidence suggest that membranes are the primary site of desiccation injury in cells and organelles (Rajasekaran and Blake, 1999). The growth and productivity of banana plant as a commercial crop in the tropical and subtropical regions of the world are adversely affected by the water stress which often is associated with the enhanced oxidative damage (Ismail et al., 2004; Chai et al., 2005; Turner et al., 2007). The development of methods to induce stress tolerance in plants is vital and still receives considerable attention. Approaches taken to develop stress-tolerant plants have included genetic engineering (McKerise et al., 1988), traditional breeding (Vettakkorumakankav et al., 1999), *in vitro* selection, and the use of growth regulators (Baninasab and Ghobadi, 2011; Senaratna et al., 2000). Salicylic acid (SA) is considered to be plant hormone-like

substance which plays an important role in the regulation of plant growth and development, seed germination, fruit yield, glycolysis, flowering, and heat production in thermogenic plants (Klessig and Malamy, 1994). Ion uptake and transport (Harper and Balke, 1981), and the rates of photosynthesis, stomatal conductance, and transpiration (Khan et al., 2003) could also be affected by the application of SA. The impact of different levels of SA at pre – harvest stage on yield and quality of cut rose was investigated by Hashemabadi and Zarchini (2010). They reported that the highest vase life was obtained with SA treatments. The role of SA in defense mechanisms in plants under biotic and/or abiotic stress suggests that it could also alleviate drought stress in plants (Senaratna et al., 2000). Exogenous application of SA was reported to have effects on a wide range of physiological processes, including increased cold tolerance of germination in pepper (Korkmaz, 2005), chilling tolerance in cucumber (Kang and Saltveit, 2002) and in maize (Janda et al., 2000), salinity tolerance in barley (El- Tayeb, 2005), improved heat shock tolerance in mustard (Dat et al., 1998), and a decrease

in the inhibitory effects of drought stress on tomato and bean (Senaratna *et al.*, 2000), and wheat (Sakhabinova *et al.*, 2003). SA is known to play an important role in modulating the redox balance across membranes, thereby counteracting the negative effects of reactive oxygen species (ROS) generated by oxidative stress (Yang *et al.*, 2004) by increasing the activity of anti-oxidant enzymes such as superoxide dismutase (Singh and Usha, 2003). Salicylic acid (SA) is known as an endogenous growth regulator of phenolic type distributed in a wide range of plant species, which induces biotic and abiotic stress tolerance in crops (Conrath *et al.*, 2002; Nowak and Pruski, 2004; Pal *et al.*, 2006; Hayat *et al.*, 2007; Horvath *et al.*, 2007; Janda *et al.*, 2007; Radwan *et al.*, 2008; Sakhanokho and Kelley, 2009; Joseph *et al.*, 2010; Ghorbani Javid *et al.*, 2011). Review of the literatures also demonstrated that SA affects other physiological processes in plants such as growth, photosynthesis, uptake of ions, heat production, flowering and ethylene production (Raskin, 1992; Hayat *et al.*, 2007; Joseph *et al.*, 2010; Ghorbani Javid *et al.*, 2011). SA was reported to induce an increase of *in vitro* regeneration frequency in *Hibiscus* plants (Sakhanokho and Kelley, 2009). Kogel and Langen (2005) asserted that the manner of SA in the induction of stress tolerance may alter when dicot and monocot plants are compared. The SA has been reported to induce water stress tolerance in *Satureja hortensis* (Yazdanpanah *et al.*, 2011). Agarwal *et al.* (2005) demonstrated the enhanced chlorophyll levels and relative water content as well as the lessened hydrogen peroxide (H₂O₂) and lipid peroxidation when the wheat leaves were treated with SA under mild water stress conditions. There are conflicting reports concerning the SA application did not mitigate the negative effects of drought stress on growth of wheat (Waseem *et al.*, 2006). However, the efficiency of exogenous SA depends on multiple causes such as the species, developmental stage of the plant, the manner of application and the concentration of SA (Borsani *et al.*, 2001; Nemeth *et al.*, 2002; Pal *et al.*, 2006; Stevens *et al.*, 2006; Horvath *et al.*, 2007; Joseph *et al.*, 2010). Few methods of SA application such as soaking the seeds prior to sowing, adding to the hydroponic solutions and tissue culture media, irrigating and spraying with SA solution have been revealed to protect different plant species against abiotic and biotic stress agents (Horvath *et al.*, 2007; Sakhanokho and Kelley, 2009; Radwan *et al.*, 2008). The capability of SA in moderating hostile effects of salinity in crop plants has been reviewed by Ghorbani Javid *et al.* (2011). They concluded that SA could be a very helpful compound showing a positive effect on plants under stressed conditions. Delavari *et al.* (2010) reported that application of 0.01 mM of SA relieved the injurious effects of salinity stress in sweet basil. Tissue culture technique has emerged as a feasible and cost-effective alternative tool for developing stress-tolerant plants in recent years. This technique can operate under controlled conditions with limited space and time (Sakhanokho and Kelley, 2009), and has the potential for selection of stress-tolerant variants using a low cost laboratory set up. The impacts of various exogenous SA concentrations on *in vitro* growth and inducing salt tolerance in shoot tips of *Hibiscus* plants have also been investigated (Sakhanokho and Kelley, 2009). The SA plays an essential role in preventing oxidative damage, as lipid peroxidation is alleviated in SA treated plants under stress conditions (Horvath *et al.*, 2007; Joseph *et al.*, 2010; Ghorbani Javid *et al.*, 2011). According to our knowledge, there are no reports on the effects of SA enhancing banana tolerance to drought stress. Therefore, the objectives of this

study were to determine (1) the effect of drought stress and SA on the some morphological and physiological exchanges in banana (2) whether application of SA to banana might be a strategy for increasing the drought tolerance.

Results

Analysis of variance

A combined analysis of variance (Table 1) demonstrated a significant influence of different doses of SA on shoot tips performance under various levels of PEG induced water stress through the micropropagation media for all traits except fresh weight increase and relative water content. Table 1 also shows a separate analysis of variance carried out for each SA and PEG. The results indicated highly significant differences in shoot tips performance demonstrating noticeable effects of SA and PEG apart, although under normal conditions, the effect of SA was not significant in terms of enhanced relative water content.

Mean comparisons

Morphological responses of shoot tip cultures to SA under water stress induced by PEG

Polyethylene glycol (PEG₆₀₀₀) induced water stress caused a significant reduction in proliferation rate and fresh weight increase of shoot tips (Table 2). When subjected *in vitro* cultures to water stress by itself, the lowest proliferation rate (0.71 ± 0.11) was observed with the highest levels (3 %) of PEG. While, the shoot tips grown on the media without addition of PEG (0 %) exhibited the highest (4.25 ± 0.21) proliferation rate (Table 2). The mean fresh weight increase under water stress conditions caused by various levels of PEG indicated a reduction of around 33.8% as compared with that obtained with non – stressed (control) conditions (Table 2). Exogenous application of SA in the media without addition of PEG showed no significant difference in proliferation rate but significantly enhanced fresh weight increase of shoot tips. The highest fresh weight increase (6.57 ± 0.27 g) was recorded with the highest concentration (3 mM) of SA and the lowest value (4.35 ± 0.42 g) was observed with shoot tips grown under the control treatment (Table 2). Under all levels of PEG induced water stress, the shoot tips responded positively to supply of SA through the micropropagation medium by showing significant increase in proliferation rate and fresh weight increase (Table 2).

Plant water status (relative water contents and leaf water loss)

Relative water content (RWC %) and leaf water loss (LWL %) of shoot tips were adversely influenced by PEG (Table 3). The percentage of RWC was less in the shoot tips grown on the stressed media in comparison with the control, as the highest level (3 %) of PEG indicated the lowest percentage (50.68 ± 8.41 %) of RWC. While, the highest value (93.36 ± 1.20 %) was observed in the shoot tips grown on the control media. However, under all levels of PEG induced water stress, SA application increased the RWC either moderately or remained unchanged (Table 3). The percentage of LWL indicated the maximum increase in shoot tips treated with the highest level (3 %) of PEG. Different doses of SA applied through the micropropagation media stressed by increasing

Table 1. Analysis of variance (ANOVA) about the effect of salicylic acid on morphological and physiological responses of banana (*Musa acuminata* cv. 'Berangan', AAA) shoot tip cultures to *in vitro* water stress mediated through polyethylene glycol.

Source of variation	df	F – Values							
		PR	FWI	P	RWC	LWL	Chl	MDA	H ₂ O ₂
PEG	3	349.63**	356.22**	81.42**	263.53**	176.40**	50.45**	72.97**	192.15**
SA	3	21.98**	121.79**	38.95**	2.02 ^{ns}	13.18**	77.31**	79.32**	52.40**
PEG × SA	9	2.11*	0.76 ^{ns}	4.65**	1.39 ^{ns}	2.01*	4.61**	12.57**	8.29**
Error	32	-	-	-	-	-	-	-	-

ns, * and ** symbolize not significant and significant at the 5% and 1% levels of probability, respectively. SA = salicylic acid, PEG = polyethylene glycol, MDA = malondialdehyde, H₂O₂ = hydrogen peroxide, PR = proliferation rate, FWI = fresh weight increase (g), P = proline content (μmoles g⁻¹ FW), RWC = relative water stress (%), LWL = leaf water loss (%), Chl = chlorophyll levels (mg g⁻¹ FW), MDA = malondialdehyde (nmol g⁻¹ FW), H₂O₂ = hydrogen peroxide (μmol g⁻¹ FW)

Table 2. Impact of salicylic acid supplemented into MS medium on morphological responses of banana (*Musa acuminata* cv. 'Berangan', AAA) shoot tip cultures to different doses of polyethylene glycol induced water stress after 2 months of culture period.

SA (mM)	PEG (%)	PR (No. of shoots/ explant)	FWI (g)
0	0	4.25 ± 0.21 ^b	4.35 ± 0.42 ^d
	1	1.39 ± 0.16 ^{fg}	1.76 ± 0.06 ^{jk}
	2	0.97 ± 0.12 ^{gh}	1.26 ± 0.06 ^l
	3	0.71 ± 0.11 ^h	1.39 ± 0.14 ^{kl}
1	0	4.44 ± 0.43 ^{ab}	5.23 ± 0.35 ^c
	1	2.09 ± 0.24 ^{cd}	2.17 ± 0.36 ^{ij}
	2	1.46 ± 0.32 ^f	1.75 ± 0.22 ^{jk}
	3	0.96 ± 0.09 ^{gh}	1.71 ± 0.22 ^{jkl}
2	0	4.72 ± 0.33 ^a	6.08 ± 0.23 ^b
	1	1.98 ± 0.39 ^{de}	3.16 ± 0.29 ^{fg}
	2	2.24 ± 0.35 ^{cd}	2.84 ± 0.28 ^{gh}
	3	1.62 ± 0.19 ^{ef}	2.50 ± 0.42 ^{hi}
3	0	4.51 ± 0.30 ^{ab}	6.57 ± 0.27 ^a
	1	2.46 ± 0.38 ^c	3.80 ± 0.32 ^e
	2	1.92 ± 0.32 ^{de}	3.22 ± 0.40 ^{fg}
	3	1.38 ± 0.08 ^{fg}	3.46 ± 0.28 ^{ef}
LSD _(0.05)		0.4561	0.4837

SA = salicylic acid, PEG = polyethylene glycol, PR = proliferation rate, FWI = fresh weight increase, Within a column, values (means ± SD) marked with the same small letters (a – l) are not significantly different at the 0.05 probability level according to the LSD test. Each treatment was replicated three times (*n* = 3) with each replication having five explants.

levels of PEG caused a non significant improvement in terms of reduced percentage of LWL (Table 3).

Proline content

Exogenous application of SA did not significantly affect proline content under normal conditions (treatments without the addition of PEG to the media). Similarly, varying levels (1 to 3%) of PEG without the SA application did not change the content of proline significantly although the highest level (3 %) of PEG caused a significant increase of proline in non SA – treated shoot tips as compared with those obtained from non PEG – stressed (control) media (Table 3). When supplemented shoot tips with 3% PEG, the application of 1 mM SA through the micropropagation media significantly enhanced the proline content of explants, however; it was decreased with 3 mM SA (Table 3).

Chlorophyll

Varying doses of SA did not indicate a significant influence on chlorophyll content of explants when PEG induced water stress was not applied through the micropropagation media (Table 3). Also, when shoot tip cultures subjected to non SA – containing treatments, addition of increasing levels of PEG to the media significantly reduced chlorophyll content of

shoot tips. On the other hand, inclusion of varying doses of SA to the PEG containing media significantly alleviated the decline in the content of chlorophyll as compared with non SA – treated explants. However, higher levels of SA (2 and 3 mM) indicated no significant difference (Table 3).

Oxidative stress indices (H₂O₂ production and lipid peroxidation)

The mean values of MDA and H₂O₂ contents of shoot tips under 3 % PEG induced water stress, indicated an increase of 31.7% and 34.8%, respectively compared with those of non – stressed (control) conditions (Table 4). It is apparent from the Table 4 that increasing PEG levels by itself, up to 3 % through the media caused a significant increase in the MDA and H₂O₂ contents, while, under normal conditions (the media without the addition of PEG) the values of MDA and H₂O₂ contents caused by varying concentrations of SA were not significantly affected. The H₂O₂ content of leaves under varying levels of PEG decreased significantly with increasing the doses of SA demonstrating the positive effects of SA on relieving the hazards of oxidative stress on banana shoot tips (Table 4). Present study also showed that the amount of MDA in the shoot tips subjected to water stress was reduced in response to SA. Therefore, the lipid peroxidation caused by water stress was ameliorated by SA treatments (Table 4).

Table 3. Effect of salicylic acid supplemented into MS medium on proline accumulation [$\mu\text{mol g}^{-1}$ (FW)], relative water content [%], leaf water loss [%] and chlorophyll [mg g^{-1} (FW)] of *Musa* AAA 'Berangan' shoot tip cultures under varying levels of polyethylene glycol induced water stress after 2 months of culture period.

SA (mM)	PEG (%)	Proline ($\mu\text{mol g}^{-1}$ FW)	RWC (%)	LWL (%)	Chl (mg g^{-1} FW)
0	0	6.78 ± 0.44 ^d	93.36 ± 1.20 ^a	11.00 ± 0.60 ⁱ	49.03 ± 2.12 ^{cd}
	1	7.53 ± 1.30 ^{cd}	77.82 ± 1.30 ^b	34.30 ± 4.50 ^{def}	37.62 ± 6.44 ^f
	2	7.40 ± 0.42 ^{cd}	68.62 ± 5.74 ^{de}	36.82 ± 5.01 ^{de}	28.19 ± 2.75 ^g
1	3	8.04 ± 0.27 ^c	50.68 ± 8.41 ^g	54.81 ± 8.31 ^a	20.28 ± 1.48 ^h
	0	7.09 ± 0.33 ^d	93.47 ± 0.64 ^a	11.22 ± 1.48 ⁱ	52.53 ± 4.86 ^{abc}
	1	9.16 ± 0.32 ^b	73.05 ± 1.73 ^{bcd}	24.23 ± 3.12 ^{gh}	48.03 ± 2.34 ^{cd}
2	2	9.76 ± 0.30 ^{ab}	67.71 ± 4.32 ^e	35.98 ± 2.13 ^{de}	40.97 ± 2.20 ^{ef}
	3	10.37 ± 0.59 ^a	52.65 ± 4.06 ^{fg}	47.65 ± 8.22 ^b	38.00 ± 2.02 ^f
	0	6.82 ± 0.29 ^d	93.37 ± 1.64 ^a	11.10 ± 0.73 ⁱ	56.58 ± 3.48 ^{ab}
3	1	9.76 ± 0.52 ^{ab}	73.41 ± 1.84 ^{bcd}	22.33 ± 3.27 ^{gh}	50.39 ± 1.91 ^{cd}
	2	10.16 ± 0.13 ^a	71.97 ± 2.85 ^{cde}	30.89 ± 1.42 ^{ef}	49.95 ± 1.01 ^{cd}
	3	10.52 ± 0.47 ^a	56.15 ± 3.25 ^{fg}	43.85 ± 3.69 ^{bc}	46.04 ± 4.32 ^{de}
3	0	6.73 ± 0.44 ^d	92.83 ± 1.16 ^a	10.91 ± 0.14 ⁱ	57.45 ± 3.97 ^a
	1	10.00 ± 0.44 ^{ab}	75.18 ± 1.18 ^{bc}	18.76 ± 2.70 ^h	51.93 ± 2.49 ^{bc}
	2	10.46 ± 0.64 ^a	73.76 ± 2.87 ^{bcd}	28.58 ± 1.29 ^{fg}	51.05 ± 3.47 ^{cd}
3	3	10.35 ± 0.17 ^a	58.06 ± 2.24 ^f	40.65 ± 2.70 ^{cd}	48.04 ± 3.13 ^{cd}
	LSD (0.05)	0.8524	5.672	6.483	5.468

SA = salicylic acid, PEG = polyethylene glycol, RWC = relative water content, LWL = leaf water loss, Chl = chlorophyll levels. Within a column, values (means ± SD) marked with the same small letters (a – i) are not significantly different at the 0.05 probability level according to the LSD test. Each treatment was replicated three times ($n = 3$) with each replication having five explants.

Table 4. Influence of salicylic acid supplemented into MS medium on oxidative stress (MDA accumulation [nmol g^{-1} FW] and H_2O_2 contents [$\mu\text{mol g}^{-1}$ FW]) of banana (*Musa acuminata* cv. 'Berangan', AAA) shoot tip cultures caused by various levels of polyethylene glycol induced water stress after 2 months of culture period

SA (mM)	PEG (%)	MDA (nmol g^{-1} FW)	H_2O_2 ($\mu\text{mol g}^{-1}$ FW)
0	0	26.77 ± 4.88 ^{gh}	16.03 ± 1.09 ^f
	1	42.57 ± 5.61 ^{cd}	21.53 ± 2.32 ^e
	2	60.67 ± 2.55 ^b	37.51 ± 1.47 ^b
1	3	84.57 ± 12.49 ^a	46.03 ± 3.75 ^a
	0	27.77 ± 5.40 ^{fgh}	15.23 ± 1.13 ^f
	1	31.15 ± 3.43 ^{efg}	16.13 ± 0.78 ^f
2	2	35.10 ± 3.28 ^{def}	30.77 ± 3.34 ^{cd}
	3	46.24 ± 2.91 ^c	33.55 ± 4.27 ^c
	0	26.41 ± 3.20 ^{gh}	14.90 ± 0.97 ^f
3	1	23.15 ± 1.47 ^h	14.90 ± 0.46 ^f
	2	28.31 ± 3.36 ^{fgh}	28.14 ± 3.38 ^d
	3	38.37 ± 4.35 ^{de}	29.28 ± 2.20 ^d
3	0	25.87 ± 2.67 ^{gh}	14.21 ± 0.33 ^f
	1	20.85 ± 1.41 ^h	14.31 ± 1.59 ^f
	2	28.21 ± 2.35 ^{fgh}	24.27 ± 2.80 ^e
3	3	38.33 ± 1.52 ^{de}	23.30 ± 1.39 ^e
	LSD (0.05)	7.639	3.809

SA = salicylic acid, PEG = polyethylene glycol, MDA = malondialdehyde, H_2O_2 = hydrogen peroxide, Within a column, values (means ± SD) marked with the same small letters (a – h) are not significantly different at the 0.05 probability level according to the LSD test. Each treatment was replicated three times ($n = 3$) with each replication having five explants.

Discussion

The positive effect of SA on morphological attributes such as multiplication rate and fresh weight of shoot tips is of interest, as some reports on *in vitro* water stress tolerance studies suggest that multiplication rate and fresh weight not only adversely affected by water stress but also positively correlate with drought tolerance (Mohamed et al., 2000; Gopal and Iwama, 2007). In the current study, morphological and physiological observations indicated that the stress caused by PEG was ameliorated when the shoot tips were supplemented with the exogenous SA application through the

micropropagation media. However, the improving effect of SA is not constantly evident concerning drought tolerance of plants (Horvath et al., 2007). Supplying of maize plants with 0.5 mM SA reduced their drought tolerance (Nemeth et al., 2002), however, SA treated wheat plants under water stress overcame the hostile effects of stress (Hamada, 1998; Sakhabutdinova et al., 2003). Senaratna et al. (2000) reported that the effective dose of SA for alleviating the injury of tomato and bean plants under water stress, ranged from 0.1 to 0.5 mM. Consequently, the efficiency of exogenous SA in relieving of hazards of stresses depends on some factors such as the plant species, the manner of application and the concentration of SA (Borsani et al., 2001; Nemeth et al., 2002; Pal et al., 2006; Stevens et al., 2006; Horvath et al.,

2007; Joseph et al., 2010). This study demonstrated that the highest levels (3 %) of PEG caused a reduction of about 54.3% in RWC% of explants as compared with those of the non – stressed conditions. However, SA application through the culture media subjected to PEG induced water stress, increased the water maintenance in the shoot tips (pointed out as enhanced RWC % and reduced LWL %). The enhanced percentage of RWC in response to increasing doses of SA, under stressed conditions, has been reported in maize (Levent Tuna et al., 2007) and wheat (Agarwal et al., 2005). In the current investigation, only in the water stressed shoot tips, SA treatments enhanced proline content which is in agreement with those reported in *Ocimum basilicum* by Delavari et al. (2010). These observations interestingly propose that proline could be an influential component in SA induced protective reaction of banana plants in response to water stress leading to a relief of hostile effects of stress agent. The present study confirmed previous observations and reports that application of SA under stressed conditions improved plants performance in terms of enhanced chlorophyll contents (Agarwal et al., 2005; Hayat et al., 2007; Levent Tuna et al., 2007; Radwan et al., 2008; Delavari et al., 2010). A significant decrease in the level of reactive oxygen species (ROS) production was noticed when SA treatments were applied to the micropropagation media in combination with PEG. Interestingly, banana shoot tips treated with SA accumulated less H₂O₂ content compared to control plants. This suggests that SA may also play an important role in inducing tolerance to oxidative stress conditions in banana which is in conformity with Agarwal et al. (2005) in the case of wheat genotypes. Lipid peroxidation was examined by estimating malondialdehyde (MDA) content in the shoot tips. Since the water stress is known to induce oxidative stress in banana (Chai et al., 2005), the PEG – treated shoot tips exhibited high rate of lipid peroxidation. Totally, shoot tips treated with SA accumulated less H₂O₂ and MDA contents compared to control plants under water stressed conditions, suggesting that SA could possess an important role in inducing tolerance to oxidative stress conditions in banana. This is supported by the findings of Agarwal et al. (2005) who mentioned that SA treatments of wheat leaves under water stress conditions, resulted in reduced amounts of MDA. The present study also confirmed the observations reviewed by Joseph et al. (2010) that the most effective role of SA in alleviating the hazards of stresses was recorded at the lower (up to 1 mM) doses, whereas, the mean comparison of most morphological and physiological traits of shoot tips of banana (*Musa acuminata* cv. ‘Berangan’, AAA) obtained with the higher SA concentrations (above 2 mM), were not significant.

Materials and methods

PEG induced water stress and SA application through the micropropagation medium

Micropropagation medium consisted of the MS (Murashige and Skoog 1962) – based tissue culture medium containing 30 g L⁻¹ sucrose and 22.2 μM benzyl aminopurine (BAP), solidified with 2.8 g L⁻¹ gelrite was prepared. Osmotic shock in the reference medium was induced by adding varying levels (0, 1, 2 and 3 %) of PEG₆₀₀₀ to the micropropagation media. After adjusting the pH to 5.6, all media containing varying levels of PEG were autoclaved at 121°C for 20 min. Then, appropriate volumes from a filter – sterilized stock solution of SA were added to the media when the reference media were just above the solidification temperature

(approximately 50°C) to reach the various doses (0, 1, 2 and 3 mM) of SA required for treatment.

Preparation of plant material and in vitro culturing

Shoot tips of 10 – 12 mm with nearly half shoot and corm tissue were aseptically detached from shoot clumps of micropropagated cultures of banana (*Musa acuminata* cv. ‘Berangan’, AAA) and then, sheathing leaves enclosing each other (pseudo stem tissue) were removed consecutively as the explants were enveloped by 3 to 5 sheathing leaves. Eventually, the extracted shoot tips of almost uniform size were inoculated in bottles provided with 50 ml micropropagation medium containing varying levels of PEG (0, 1, 2 and 3 %) and SA (0, 1, 2 and 3 mM) as mentioned above. Cultures were maintained in growth chambers at 28 ± 2°C, under 16 – h photoperiod provided by fluorescent lamps for two months.

Morphological measurements

Two months after inoculation of shoot tips onto various SA doses containing micropropagation media under different levels of PEG induced water stress, proliferation rate was determined by counting all shoots regenerated per explant. Since the fresh weight of the initial explants treated for experiment need to be as stable as possible, the mean fresh weight of five shoot tips for each replication was considered 0.8 ± 0.1 g at the starting point, then the fresh weight of the proliferated shoots two months after culture on the micropropagation media containing various levels (0, 1, 2 and 3 mM) of SA and PEG (0, 1, 2 and 3 %) minus shoot tips fresh weight at the starting point was served as fresh weight increase.

Physiological observations

Physiological attributes such as the relative water content, leaf water loss, proline content, chlorophyll levels, MDA formation and H₂O₂ content were assessed using the leaf samples removed from developing shoot tips on the treatments containing media after 2 months of culture.

Relative water content and leaf water loss

Measurement of the relative water contents (RWC %) was performed according to Mata and Lamattina (2001) and was calculated using the following equation:

$$\text{Relative water content (\%)} = \frac{FW - DW}{TW - DW} \times 100$$

Leaf water loss (LWL) was evaluated according to the method of Xing et al. (2004) and then, was calculated using the following formula:

$$\text{LWL (\%)} = \frac{W1 - W2}{W1} \times 100$$

Measurement of proline content and chlorophyll levels

The Proline content was measured according to the method described by Bates et al. (1973). Fresh leaf samples (300 mg) were removed from *in vitro* regenerating shoot tips, then, chlorophyll contents were extracted with 90% acetone and calculated using the formula given by Porra (2002). Chls *a* + *b* (mg.g⁻¹ FW) = [8.02 × (A663) + 20.20 × (A645)] × V/1000 × W. Where V, W, FW, A663 and A645 represented volume of the extract (ml), weight of fresh leaves (g), fresh weight,

absorbance values read at 663 and 645 nm wavelengths, respectively.

Oxidative stress indices (ROS production and MDA accumulation)

The content of malondialdehyde (MDA) which is a product of lipid peroxidation was measured by the thiobarbituric acid (TBA) according to Wang et al. (2009) and was calculated on a fresh weight by $(\text{nmol MDA g}^{-1} \text{FW}) = 6.45 (\text{OD}_{532} - \text{OD}_{600}) - 0.56 (\text{OD}_{450}) \times 1000$. Where, OD_{450} , OD_{532} and OD_{600} represent absorbance values read at 450, 532 and 600 nm wavelengths, respectively. Hydrogen peroxide (H_2O_2) was measured according to method of Velikova and Loreto (2005).

Statistical analysis

The factorial experimental design with one cultivar, four SA doses (0, 1, 2 and 3 mM) and four PEG levels (0, 10, 20 and 30 g L^{-1}) were arranged in a completely randomized design (CRD) with three replications ($n = 3$). Data of morphological and physiological indices were subjected to an analysis of variance (ANOVA) using a SAS statistical program. Furthermore, significant differences among the mean values of treatments were compared by least significant difference (LSD) test method at $P \leq 0.05$ using the MSTAT-C computer program.

Conclusion

Improving some physiological and morphological attributes of SA – treated shoot tip cultures of *Musa acuminata* cv. 'Berangan' under water stressed conditions donates a protective reaction aimed to ameliorate the hostile effects of stress agent (PEG) on banana. Therefore, exogenous SA could be applied as a potential growth regulator to improve banana water stress tolerance.

Acknowledgements

The authors acknowledge the financial support of Research University Grant Scheme (RUGS) – UPM, University Putra Malaysia for the project.

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