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Aseptic Growth Attributes of PEG-Stressed and BBTV-Infected Plants of Banana

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Abstract: ***Aseptic multiplication of banana is a major tool for rapid mass production of pathogen free plants. In this experiment, comparative micropropagation growth was assessed in polyethylene glycol (PEG) stressed and Banana bunchy top virus (BBTV) infected plantlets of banana (Musa spp.)cv., Basrai under aseptic conditions. This comparative study was conducted, when healthyand BBTV-infected plantlet were multiplying [MS_2 (8 mg L^{-1} BA, 2 g L^{-1} phytagel)] at 2nd sub-culture stage. They were transferred to fresh media for 3rd subculture and labelled as MS_2 (control),MS_{2b}(BBTV-infected plantlets) and MS_{2c} (MS_2 + 5% PEG).After 6-weeks of culture, lowest micropropagation efficiency was observed in PEG stressed cultures (3.25 ± 0.408 plantlets) than BBTV infected (4.50 ± 0.289 plantlets) and control cultures (5.75 ± 1.555 plantlets). Reducing sugars, proline, glycinebetaine contents and total carotenoids were increased ($p < 0.05$) in PEG stressed as well as BBTV infected plantlets, while total sugars, proteins and nitrates were decreased significantly.Phenolics were increased but relative water contents decreased with the decrease in shoot biomass in PEG stressed and BBTV infected plantlets than control healthy plantlets($p < 0.05$). This instability of organic and in-organic attributes in the micro-propagated plantlets reflects relative tolerance of plant growth in banana cultivar Basrai against the applied abiotic (PEG) and biotic (BBTV) stresses.***

Keywords: *Musa* spp., micro-propagation, BBTV, PEG, reducing sugars, total carotenoid, reducing sugars, nitrate contents.

1. Introduction

Edible banana (*Musa* spp.) is delicious table-fruit with abundant carbohydrates (25%) and equally beneficial from infants to old man. It is one included among the other important staple food crops and being a valuable crop for domestic trade. Rate of world's production of banana has reached to~ 44 tons hec^{-1} (Frison*et al*., 1997; FAO, 2005). This production rate is decreasing with the passage of time because of increasing environmental factors like as deficiency of water, increasing salinity and attacks by nematodes, bacteria, fungal and viral disease especially banana bunchy top virus (BBTV).All of these stresses are limiting physiological aspects of plants because of reduction in photosynthetic leaf areaandfinally results in banana yield reduction (Mobambo*et al*., 1993).

Expansion of banana fields is also limitedbecause it's decreasing yields by a number of biotic and abiotic stresses. Even banana fields are replacing with other crops by the farmers. Reason is thatnon-availability of healthy plant materialto the farmers for cultivation or replacement of old banana farms with new healthy nursery. *In-vitro*micro-propagation from newly emerging micropropagation has prompted farmer's interest to cultivate asepticbanana plantlets (Ortiz and Vuylsteke,1996; Tenkouano*et al*., 1998a). The plant propagation is most efficient to multiply plantlets in very short time within little space. This system is also considered as a best plat-form to study the impacts of applied stresses of salinity or drought or even biotic stresses on cultured plantlets (Haq*et al*., 2011, 2014).

However, abiotic and biotic stresses are playing around the life cycle of plants including BBTV and water deficient conditions are limiting it's both vegetative as well as reproductive or yield growth. These factors are increasing day by day, while many crops have got resistance mechanisms by evolution in their metabolics to combat against water deficit conditions (Sadeghian and Yavari,2004). At the moment, not a single edible banana cultivar has drought resistance, which might be recommended to farmers for cultivation on water deficit agriculture areas. Meanwhile, polyethylene glycol (PEG) is one could be used to induces waterstress in balance plant nutrient culture (Ruf*et al*.,1967; Kaufman and Eckard, 1971). It decreases the availability of water to plant tissues by reducing osmotic potential of the culture medium. In this way water cannot keep its needful water quantity inside the tissue even squeezed out and increases phyto-toxicity within the cells or tissues (Lawlor, 1970).

In banana fields, it is difficult to differentiate that a plant is growing under water stress or infected with banana viruses. One out of five banana viruses is BBTV has considered as lethal for banana plant while its initial symptoms are very similar to as developed by abiotic stresses and remain difficult to judge with naked eyes. Plant tissue culture is a technique, which can provide a control and homogenic plant nutrition forplants to measure theactual impacts of environmental stresses on the cell, while at a whole plant level the system could not be performing as such (Lutts*et al*., 2004).

In the light of above citation, this experiment has conducted to analysis comparative effects of water deficit stress outside the tissue plantlets and BBTV stress inside the tissue under *in-vitro* condition. Present study may be helpful in future for the improvement ofdrought resistant in banana crop and correct selection of BBTV free plants in banana fields.

2. Material and Methods

2.1 Banana micropropagation

From the open field conditions four newly emerging banana (*Musa spp*) cv., Basrai young plants were collected from both healthy and BBTV infected parent plants (Haq et al., 2009; Haq et al., 2014). Inner meristematic stem tips were

excised and washed with ethanol (90%) for 1min than stirred in 20% bleach(5.25% NaOCl) for 30 min. This sterilized explant was used for induction of shoot and then shoot multiplication according to Haq and Dahot (2007a, 2007b, 2007c) and Haq*et al.* (2011, 2014). Briefly, as shoot tips (3 - 4 mm) were divided intotwo parts and cultured on MS_1[MS_o (Murashige and Skoog,, 1962) basal salts with B5 vitamins(Gamborg*et al*., 1968), 10 mg L^{-1} BA, 15 mg L^{-1}IAA, 30.0 mgL^{-1}Lcystein, 3% sucrose and 3.60g L^{-1} phytagel] medium for 15-20-days for organogenesis. Shoot induction was carried on MS_{1a} (MS_o, 8mg L^{-1}BA and 20 mg L^{-1} L-cysteine, 1.0 g L^{-1}phytagel), while shoot micropropagation on MS_2(MS_o, 8 mg L^{-1} BA and 20 mg L^{-1} L-cystein, 2.0 g L^{-1} phytagel). Shoot multiplication culture were refreshed after every 3rd week, while 3rd subculture was subjected for PEG stress (represented as MS_{2b}) along with BBTV infected tissue (represented as MS_{2a}).

2.2 Establishment of PEG-stressed along BBTV infected cultures

On 3rd week of 2nd subculture, rapidly micro-propagating plantlets from healthy (MS_2) and BBTV infected (MS_{2a}) cultures were refreshed on same culture including one with PEG stress on healthy shoot multiplication [MS_{2b} (MS_2,5% PEG-4000)] medium.These cultures were labelled as MS_2 (control), MS_{2b} (BBTV-infected plantlets) and MS_{2c} (MS_2 + 5% PEG) and incubated for 6-weeksunder 18/6 h lightening with ~2000 lux intensityat 25 ± 1°C.The pH of each culture was adjust 5.7 to 5.8 and autoclaved at 121°C with 15 lbs for 10 min.

2.3 Measurement of Morphological Parameters

At the end of 6th week of culture, micro-propagated plantlets taken out from culture glass jars and washed with running tap-water to removed entangled medium. Plantlets were dried on filter-paper and number of plantlets per explant were counted. Shoot height and fresh shoot biomass was taken. Dry shoot biomass was measured by drying shoots at 72°C with electric oven for 72-hrs. Relative water contents (RWC) were expressed [Catsky, 1974; Turner, 1981] by using this formula - *RWC (%) = [(FM - DM)/TM] * 100*.

2.4 Measurement of Biochemical Parameters

Fresh shoots were subjected for biochemical analysis like as chlorophyll or photosynthetic pigments weredetermined by using methods of Arnon (1949) and Lichtenthaler(1987). Glycinebetaine (Grieve and Gratter, 1983) andproline (Bates *et al*., 1973) contents were determined spectrophotometrically.Total phenol contents were measured as by Ozyigit*et al*. (2007).Dry shoot material was also analyzed for various biochemical contents like as total protein contents(Bradford, 1976), total carbohydrates (Dubois *et al*., 1956; Ciha andBrun, 1978), reducing sugars (Miller, 1959) and nitrate contents(Morris and Riley, 1963) were analyzed.

2.5 Statistical Data Analysis

Data significance of treatment [each treatment with 4-replicates were arranged for one way analysis of variance (ANOVA)] was calculated by using COSTAT computer statisticalsoftware (*CoHort*software, Berkeley, USA) and DMR test at 5% level of treatment difference.

3. Results and Discussion

Banana is a delicate herbaceous plant with false stem and grow above the ground. Stem is comprised on leaf-sheaths full with water. Delay is irrigation, it shows wilting character that is very similar to symptoms developed by plants growing under abiotic stresses like as salinity, cold, high temperature as well as same to biotic stressed symptoms by initial *Banana bunchy top disease* (BBTD) infection (Haq*et al*., 2014). Both stresses cause growth inhibition and have obviously playing their harmful effects on vegetative growth and yield of banana crop. Diverse soil texture, non-availability of water and pathogen infection have negative impacts on growth of banana crop plants. Such growth retarding factors can be studied in the laboratory for identification of their precise impacts on morpho-physiological aspects of multiplying plantlets. In this experiment, young suckers of BBTV-infected banana plants and healthy banana were cultured under *in-vitro* conditions for multiplication. When micro-propagation cultures ready for 3rd sub-culture stage. At this stage, plantlets were also sub-cultured on micro-propagation medium supplemented with 5% PEG-4000 in comparison to healthy and BBTV-infected multiplying plantlets.

In nature, soil exposes its versatile characteristics to the growing plants. Some of them beneficial for plant growth while others especially balance supply of water to growing plants is not always same. When water level down than soil nutrients also have toxic impacts on plant growth both cases water deficit and soil salinity causes growth inhibition. *In-vitro* plant micro-propagation is a correct way for supply of balanced nutrition for optimal plant biomass production. This system is also suitable for study of diverse impacts of different growth retarding factors at cell level. So 3-typed plant micropropagation cultures of banana (*Musa* spp.) cv., Basrai were established like as MS_2 (healthy banana plantlets), MS_{2b} (BBTV-infected plantlets) and MS_{2c} (healthy plantlets under 5% PEG stress) for 6-weeks. Both PEG stressed and BBTV infected cultures showed a number of variations in their bio-metrics in comparison to healthy plantlets (MS_2) as shown in table 1.

Plant multiplication rate was observed maximum in MS_2 medium as already suggested by Haq and Dahot (2007b, c), over other two cultures like as MS_{2a} and MS_{2b}. Culture effect on multiplication rate was non-significant, while order of decrease in number of plantlets per explant was MS_2> MS_{2a}> MS_{2c}. The PEG stress have reduced plant multiplication rate greater than BBTV infection, while abnormal plant growth was observed among the multiplying banana plantlets in BBTV infected cultures (Fig 1). Fresh weight and dry weight of plantlets were decreased significantly ($p < 0.05$). Plant growth rate or fresh biomass has significant relationship with green photosynthetic pigments that also decreased significantly (Haq*et al*., 2008). Chlorophyll b was decreased in BBTV infected cultures like to chlorophyll a, while it slightly increases in PEG stressed plantlets. Overall decrease in total chlorophyll contents was significant.

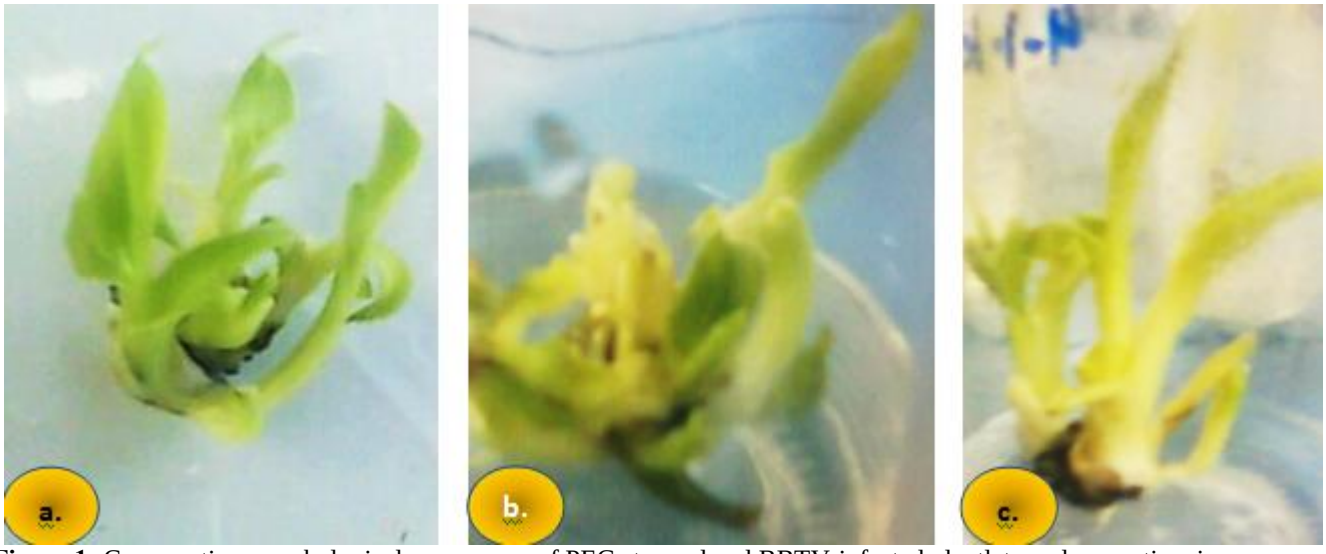

Figure 1: Comparative morphological appearance of PEG stressed and BBTV-infected plantlets under aseptic micropropagation of Banana (*Musa* spp.) cv., Basrai. a: Control MS_2 culture; b: MS_{2b} cultures with BBTV infected plantlets; c: MS_{2c} -5% PEG-4000 stressed plantlets.

The trend of coloured pigments like as total carotenoids was opposite to green or photosynthetic pigments, they were increased significantly in both PEG stressed as well as in BBTV infected plantlets (Table 1). It can be observed from figure 1, there variation in morphology of micro-propagating culture after 6-weeks. Generally, abiotic-stresses cause chloroplast-injury lead to decrease in green pigmentation. Less pigmentation, low photosynthesis results to decrease in plant vegetative growth. Water deficit stress increases the concentrations of certain free amino acids in the cells for resist against plastid's injury like as proline and glycinebetaine. In actual, both proline and photosynthetic pigments are synthesized in mesophyll cells from same precursor. Under stresses, when breakdown of photosynthetic pigments starts, well there initiation of proline synthesis also starts that could be a best indication in plants are being under stress either biotic or abiotic (Aspinal and Paleg, 1981).

Under environmental stresses, plants express certain molecular bio-markers as indicators. Like as free amino acids and carbohydrates also. Reducing sugars also increased significantly under both PEG as well as BBTV infected banana plants (Table 1). Total proteins and total carbohydrates were decreased ($p < 0.05$) in stressed plantlets. The phenolic components were also increased ($p < 0.05$). Nitrates were decreased among the stressed cultures significantly. Decrease in important plant nutrient substituents under stressed conditions causes inhibition of growth, while increase in certain components in the cell are responsible for prevention of cell injury by stressing factors. These accumulated biocomponents can be a source of carbon, hydrogen, nitrogen and energy for the recovery of inhibited physiological processes upon relax of stresses (Blum and Ebercon, 1976; Slama*et al*., 2007).All external stresses are limiting the plant growth efficiency significantly (Hartmann *et al*., 2005). Especially, abiotic stresses (salinity and drought) increases osmotic potential within the cell (Djibril*et al*., 2005; Vanden and Zeng, 2006). Biotic stresses could also be playing same when infecting the plant tissue externally, like as fungus, nematodes or carnivores, while viruses also have significant role in plant growth inhibition either by malfunctioning of metabolic biosynthesis or peliotropism in gene expression level of the plant cell.. In this experiment, almost all the parameters were considered to be affected significantly in the micro-propagating plantlets of banana (*Musa* spp.) cultivar Basrai. Both photosynthetic pigments and organic contents developed differential characteristics in the stressed cultures in comparison with the control.

4. Conclusions

Edible banana is afruit but being one among the important stable food crops, while farmers are replacing banana with other food crops because of its yield production is decreasing to 100% by BBTV infection and now deficiency of irrigation water in the rivers. Both stresses decrease its plant multiplication rate and yields because of increase in osmotic potential of the cell. Meanwhile, lot of free amino acids especially proline and glycinebetaine including other specific groups of reducing sugars and total carotenoids are increased as being stress related bio-markers. Their biosynthesis is activated by osmotic signals developed under external as well as internal stresses. These organic contents could develop specific tolerance against biotic and abiotic stresses but under viral stress is still question mark. Future layout for development resistance against specific factors depends on the expression of specific markers but virus infection based markers are not completing a task to handle related phenomena.

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Table 1: Comparative morpho-bio-chemical attributes of PEG stress to BBTV infected plantlets of banana (*Musa* spp.) cv., Basrai micro-propagated for 6-weeks at3rdsub-culture stage.

#s.	Characteristics	MS_2- control cultures	MS_{2a}- 5% PEG cultures	MS_{2b}- BBTV infected cultures	Significance @ 5%
	A. Morphological attributes				
a.	# of shoots explant^{-1}	^{a}5.75 ± 1.555	^{ab}4.50 ± 0.289	^{b}3.25 ± 0.408	ns
b.	Shoot F.Wt (g)	^{a}1.981 ± 0.002	^{b}1.635 ± 0.001	^{c}1.334 ± 0.001	***
c.	Shoot D.Wt (g)	^{a}0.581 ± 0.004	^{b}0.451 ± 0.005	^{c}0.374 ± 0.005	***
	B. Chlorophyll contents(mg g^{-1})				
a.	*Chlorophyll* a	^{a}0.127 ± 0.117	^{b}0.067 ± 0.025	^{c}0.038 ± 0.011	***
b.	*Chlorophyll* b	^{b}0.114 ± 0.005	^{c}0.070 ± 0.013	^{a}0.123 ± 0.009	***
c.	*Chlorophyll* ab	^{a}0.240 ± 0.004	^{c}0.136 ± 0.005	^{b}0.161 ± 0.004	***
d.	Total carotenoids	^{c}1.783 ± 0.002	^{b}1.952 ± 0.006	^{a}2.144 ± 0.002	***
	C. Organic contents				
a.	Total carbohydrates (mg g^{-1})	^{a}1.104 ± 0.002	^{ab}1.085 ± 0.001	^{c}1.070 ± 0.001	ns
b.	Reducing sugars (mg g^{-1})	^{c}0.547 ± 0.007	^{b}0.645 ± 0.007	^{a}0.701 ± 0.010	***
c.	Total protein contents (mg g^{-1})	^{a}1.491 ± 0.004	^{b}1.301 ± 0.003	^{b}1.273 ± 0.003	***
d.	Proline contents (mg g^{-1})	^{a}0.844 ± 0.004	^{b}0.867 ± 0.003	^{c}0.944 ± 0.003	***
e.	Glycinebetaine contents (mg g^{-1})	^{c}0.640 ± 0.002	^{b}0.776 ± 0.001	^{a}1.053 ± 0.001	***
f.	Phenolics (mol m^{-3} g^{-1})	^{c}0.271 ± 0.004	^{b}0.364 ± 0.003	^{a}0.450 ± 0.003	***
	D. in-organic contents				
a.	RWC (%)	^{a}222.25 ± 0.002	^{b}180.98 ± 0.003	^{c}142.74 ± 0.002	***
b.	Nitrates -NO_3^{-1} (µmol g^{-1})	^{a}0.162 ± 0.007	^{b}0.099 ± 0.008	^{b}0.102 ± 0.010	***

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