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Altered Response to Biotic and Abiotic Stress in Tissue Culture-Induced Off-Type Plants of East African Highland Banana (*Musa* AAA East Africa)

[1]Theodosy Msogoya and [2]Brian Grout
[1]Postgraduate School, Writtle College, Chelmsford CM1 3RR, UK
[2]Life Sciences, University of Copenhagen, 2630-Taastrup, Denmark

Abstract: This study was conducted to investigate black sigatoka disease and drought stress responses of off-type plants derived from shoot-tip micropropagation of East African highland banana (*Musa* AAA East Africa) landrace Uganda. Results showed that the off-type plants were more ($p<0.05$) tolerant to black sigatoka disease with the infection index of 17.5% compared to 30.1 and 22.8% of the micropropagation (MP) derived phenotypically normal plants and Conventional Propagation (CP) derived plants with no tissue culture history in their ancestry, respectively. On the contrary, the off-type plants were more ($p<0.05$) vulnerable to water stress with leaf senescence of 87.7% at soil water deficit of 630 millibars. The leaf senescence of the MP and CP derived plants at the same soil moisture deficit was 79.5 and 66.7%, respectively. During this stress period each off-type plant produced one sucker, while the true-to-type plants were unable to do so. Leaf structural analysis revealed that the off-type plants had higher ($p<0.05$) stomatal density of 16.0 mm^{-2} of the upper leaf surface. Conversely, the MP and CP derived plants had each 12.3 and 11.0 stomata mm^{-2} of the leaf upper surface. Similarly, the off-type plant leaves were more hydrophobic with higher ($p<0.05$) epicuticular waxiness of 684.6 $\mu g\ cm^{-2}$. The epicuticular wax content of the MP and CP derived plant leaves was as low as 646.2 and 647.7 $\mu g\ cm^{-2}$, respectively. The water stressed off-type plant leaves exhibited higher ($p<0.05$) membrane damage with ion leakage of 168.2 $\mu S\ cm^{-1}$ compared to 139.7 and 136.8 $\mu S\ cm^{-1}$ of the MP and CP derived plants. Moreover, the water stressed leaves of the off-type plants had enhanced total antioxidant activity of 5.17 M trolox equivalent per milligram proteins, whereas the total antioxidant activity of the MP and CP derived plant leaves was as low as 3.76 and 3.67 M trolox equivalent per milligram proteins, respectively.

Key words: Black sigatoka, drought stress, stomatal density, cell membrane injury, antioxidant activity, somaclonal variation, East African highland banana

INTRODUCTION

A controlled plant response to tissue culture-mediated stress may result in an enhanced plant protection against adverse biotic and abiotic environment (Cassells and Curry, 2001). Good examples include, *in vitro* derived off-type banana plants with tolerance to yellow sigatoka (*Mycosphaerella musicola* Lench) (Trujillo and Garcia, 1996) and somaclonal variants from African plantains with tolerance to black sigatoka (*Mycosphaerella fijiensis* Morelet) (Nwauzoma *et al*., 2002). Also reported are somaclonal variants of wheat with tolerance to high aluminium level (Carver and Johnson, 1989) and Cavendish banana cv. Grand nain with tolerance to mild winter (Damasco *et al*., 1996). On the contrary, a high incidence of banana streak virus was reported in micropropagated plantain cv. Superplatano (Krikorian *et al*., 1999) and severe leaf spot diseases (*Ascochyta rhei* and *Ramularia rhei*) in micropropagated rhubarb line CP49 (Zhao, 2004).

Tissue culture-mediated stress enhances the production of free radicals, which attack unsaturated lipids in the cell membrane and other macroelements such as nucleic acids and proteins (Benson, 2000; Campos *et al*., 2003; Johnston *et al*., 2006). In response to this stress, plants produce more antioxidants to defend themselves against the toxicity of reactive oxygen species and their secondary products (Prochazkova *et al*., 2001). In addition, tissue culture conditions often affect the formation of leaf waxiness, number of stomata and guard cell functioning (Blanke and Belcher, 1989; Jonathan *et al*., 1997; Majada *et al*., 1998). Changes in cell membrane stability, antioxidant activity, leaf waxiness and stomatal density affect plant responses to *ex vitro* biotic and abiotic stresses (Marin *et al*., 1988; Kirdmanee *et al*., 1996; Busogoro *et al*., 2004).

Shoot-tip micropropagated East African highland banana (*Musa* AAA East Africa) landrace Uganda exhibited high incidence of off-type plants. This instability could be utilised as a tool for the improvement

Corresponding Author: Msogoya Theodosy, Postgraduate School, Writtle College, Chelmsford CM1 3RR, UK

of landrace Uganda if the agronomic performance of the off-type plants were known. The agronomic improvement of landrace Uganda, like all landraces of East African highland bananas, is required to increase plant tolerance to various biotic and abiotic stresses (Rukazambuga *et al*., 1998). The objective of this study was to investigate the tolerance to drought stress and black sigatoka disease of tissue culture-derived off-type plants of landrace Uganda.

MATERIALS AND METHODS

Description of study areas and plant materials: East African highland banana landrace Uganda with no micropropagation history in their ancestry was micropropagated at Sokoine University of Agriculture according to Maerere *et al*. (2003). Briefly, initial explants of 2.0×2.5×50 mm were isolated from sword suckers, surface-sterilised in ethanol (96% v/v) for 2 min and disinfected for 20 min in 3.5% (m/v) sodium hypochloride. The cubes were rinsed three times in sterile distilled water and aseptically dissected to remove bleached tissues. A final shoot tip explant of about 10×10×15 mm consisted of a portion of the corm, 3-4 leaf primordia and meristematic dome. The growth media comprised Murashige and Skoog (1962) salts and vitamins at 4.4 g, L-cystein at 40.0 mg, 6-benzylaminopurine at 6.0 mg, indole-3-acetic acid at 2.0 mg, sucrose at 30.0 g and agar at 8.0 g L^{-1}. The cultures were initially incubated in darkness at 26-30°C for 4 weeks. The proliferation was carried out using the same growth media and temperatures and a photoperiod of 16 h day^{-1} supplied by cool white fluorescent tubes. Successive subcultures were carried out at an interval of 4 weeks.

The shoots were transferred to a rooting media at the fifth subculture. The rooting media consisted of MS salts and vitamins at 4.4 g, L-cystein at 40.0 mg, naphthaleneacetic acid at 2.0 mg, sucrose at 30.0 g and agar at 8.0 g L^{-1}. Rooted plantlets at about 5 cm tall were acclimatised in coconut peat media for 4 weeks under shades of 50 and 30%, respectively. The *in vitro* suckers were planted in the field at Sokoine University of Agriculture in May 2004. Planting holes of 100×100×100 cm dimension spaced at 300 m apart were prepared and filled each with 60 L of farmyard manure. The crop received optimal management, including weeding, irrigation during dry season, application of farmyard manure in subsequent years and desuckering to maintain four plants per stool. Off-type plants were detected and 30 suckers each of the off-type banana (treatment), micropropagation (MP) derived phenotypically normal banana (control 1) and Conventional Propagation (CP) derived banana with no tissue culture history in its ancestry (control 2) were collected and planted in new plots in May 2006.

Black sigatoka disease: Black sigatoka disease infection under natural inoculation conditions was assessed using 30 field-grown plants of the off-type, MP and CP derived plants in April 2007. The disease was evaluated during the rainy season using fully expanded leaves based on 0-6 severity scale according to Gauhl *et al*. (1993). In this scale, 6 was scored when 51-100% of the total leaf area died as a consequence of the disease infection and 0 when no disease symptom was visually observed on the leaf. The disease infection index was calculated according to Orjeda (1998).

Drought stress responses: Suckers of the off-type, MP and CP derived plants were collected from the field-grown parents and planted in 20 L containers in February 2007. The growth media consisted of forest soil, farmyard manure and rice husk at 3:2:1 (v/v), respectively. Thirty container plants at 1.5 m tall stage each of the off-type, MP and CP derived banana were transferred to a plastic tunnel with 30% shade screen and average day temperatures, relative humidity and light of 26°C, 68% and 2,000 lux, respectively. In April 2007, the plants were water-stressed by withholding irrigation until the soil moisture level monitored by a tensiometer (Eijkelkamp Agrisearch, The Netherlands) reached 630 millibars. The plant water stress response was evaluated based on the decrease in pseudostem diameter, leaf senescence and sucker production. The pseudostem diameter at 65 cm high was measured using a vernier caliper, whereas leaf senescence was scored when at least a quarter of one or both side(s) of the margin length was yellow. Suckers were counted as soon as they emerged out to the soil surface.

Stomatal density: The leaf stomatal density was determined from banana cigar leaves according to Marin *et al.* (1988). Stomatal density was calculated as the number of stomata per unit leaf area of the leaf lower and upper surfaces.

Leaf epicuticular waxiness: The wettability of leaf upper and lower surfaces was estimated based on static contact angle according to Koch *et al*. (2006). The contact angle between water droplet and leaf surface was measured using a protractor. Similarly, leaf waxiness was determined gravimetrically according to Kim *et al.* (2006). Leaf waxiness was expressed in microgram per leaf area (total of upper and lower surfaces).

Electrolyte leakage: Electrolyte leakage was determined using fully expanded leaves according to Saneoka *et al.* (2004). A pilot study was carried out to determine mannitol concentration that resulted in a highest leaf electrolyte leakage. Ten millilitres of mannitol solution at 0.1, 0.2, 0.3, 0.4 and 0.5 M was each dispensed in a test tube. The initial conductivity of the mannitol solutions in the test tubes was measured at 25°C using a conductimeter (Hanna Instruments Limited). Six leaf discs from washed leaves were dipped in the mannitol solution in each test tube and incubated at 26°C for 3.0 h on a rotary shaker at 150 rpm. The final conductivity of the solution was measured again and the electrolyte leakage was calculated as the difference between the final and initial conductivity of the mannitol solution. The highest ion leakage was observed at 0.3 M of mannitol. Leaf discs of equal surface area from the off-type, MP and CP derived plants were dehydrated at 0.3 M mannitol solution as described above. Electrolyte leakage was measured as described above and the ion leakage was expressed in microsiemen per centimetre.

Antioxidant activity: Cigar leaves from the off-type, MP and CP derived plants were collected in the morning, wrapped in moist tissue papers and air-transported to the United Kingdom. The leaves were kept for 5 days at 5°C in a refrigerator to undergo gradual water stress. Antioxidants were extracted from the stressed leaves according to CRYMCEPT (2005) and total antioxidant activity was determined according to Re *et al.* (1999). The total antioxidant activity was determined using a trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) standard curve and expressed as trolox equivalent. To express antioxidant activity per milligram of proteins, total protein content from the antioxidant extract above was determined by measuring its absorbance at 260 and 280 nm in the quartz cuvette. To exclude nucleic acids in the extract, total protein content at 280 nm was calculated based on Christian and Warburg equation (Caprette, 1995) as follows:

$$P = (1.55\times A280) - (0.76\times A260)$$

Where:
P = Total water-soluble protein content (mg mL^{-1})
A280 = Water-soluble protein absorbance at 280 nm
A260 = Nucleic acid absorbance at 260 nm

Data analysis: Data analysis was performed using SPSS 15.0 (SPSS[R], 2006). Percentage data were arcsine-transformed to normalise their distribution. Data were subjected to one-way parametric and non parametric ANOVA based on F-test and Kruskal-Wallis test, respectively (Zar, 1997). Multiple means comparison was carried out based on Tukey honest significant difference (Tukey-HSD) test ($p<0.05$).

RESULTS

Plant tolerance to black sigatoka disease and water stress: The off-type banana plants were more ($p<0.05$) tolerant to black sigatoka disease than the MP and CP derived plants (Table 1). Moreover, the black sigatoka infection index of the MP derived plants was significantly higher ($p<0.05$) than that of the CP derived plants (Table 1). On the other hand, the off-type plants suffered more ($p<0.05$) from the water stress with higher ($p<0.05$) decrease in pseudostem diameter and increase in leaf senescence. The off-type plants also produced suckers during the drought stress, while none of the true-to-type plants did so during the same water stress.

Cellular mechanisms underlying plant biotic and abiotic stress responses: The off-type plants had a bigger ($p<0.05$) number of stomata on the leaf upper surface than the MP and CP derived plants (Table 2). They also had higher ($p<0.05$) stomatal density on the lower leaf surface than the MP derived banana plants. The off-type plant leaves were more ($p<0.05$) hydrophobic with higher ($p<0.05$) epicuticular wax content than the MP and CP derived plants. Water-stressed leaves of the off-type plants exhibited increased cell membrane damage with higher ($p<0.05$) electrolyte leakage than the true-to-type

Table 1: Black sigatoka disease infection and drought stress responses of the off-type banana plant of landrace Uganda (±SEM)

		Drought stress responses		
Plant type	Black sigatoka disease infection index (%)	Pseudostem diameter reduction (%)	Yellow and dead leaves per plant (%)	No. of suckers per plant
Off-type plants	17.5 ± 0.9^{a}	17.4 ± 0.5^{b}	87.7 ± 3.3^{c}	0.8 ± 0.2^{b}
MP plants	30.1 ± 4.5^{c}	16.0 ± 0.6^{ab}	79.5 ± 4.4^{b}	0.0 ± 0.0^{a}
CP plants	22.8 ± 2.0^{b}	14.9 ± 0.9^{a}	66.7 ± 5.1^{a}	0.0 ± 0.0^{a}

a, b, c: Numbers bearing the same superscript letter(s) within the column are insignificantly ($p<0.05$) different according to Tukey-HSD test, SEM: Standard error of the mean (n = 30)

Table 2: Stomata density, epicuticular wax content, electrolyte leakage and antioxidant activity of stressed leaves of the off-type plants (±SEM)

Variables	Sample size	Off-type	MP plants	CP plants
No. of stomata (mm^{-2}) (Lower)	80	78.20±1.3^b	78.80±1.5^b	72.00±1.0^a
No. of stomata (mm^{-2}) (Upper)	80	16.00±0.3^b	12.30±0.2^a	11.00±0.1^a
Contact angle (°) (Lower)	40	63.00±0.8^b	62.90±1.2^b	50.20±1.4^a
Contact angle (°) (Upper)	40	63.80±0.7^b	42.00±1.5^a	45.50±1.0^a
Leaf waxiness ($\mu g\ cm^{-2}$)	20	684.60±16.1^b	646.20±0.9^a	647.70±0.9^a
Electrolyte leakage ($\mu S\ cm^{-1}$)	20	168.20±4.7^b	139.70±3.9^a	136.80±3.9^a
Antioxidant activity (M Trolox mg^{-1} proteins)	20	5.17±0.37^b	3.76±0.25^a	3.67±0.20^a

$^{a,\,b}$: Numbers bearing the same superscript letter(s) within the row are insignificantly ($p<0.05$) different according to Tukey-HSD test. SE: Standard error of the mean

plants. The water-stressed leaves also produced more ($p<0.05$) antioxidants than the true-to-type plant leaves.

DISCUSSION

Plant response to black sigatoka disease and drought stress: The off-type plants were more tolerant to black sigatoka disease compared with the MP and CP derived plants. The black sigatoka disease incidence is considered to be low, mild and severe when the infection index is less than 20, 21-50 and 51-100%, respectively (Orjeda, 1998). Almost all East African highland bananas are susceptible to black sigatoka disease (Tushemereirwe, 1987) with popular landraces such as Bukoba and Embwailuma having infection indices of 24.4 and 30.5%, respectively (Msogoya *et al*., 2006). Tissue culture derived somaclones with increased resistance to diseases have also been reported, including off-type African plantain cv. Agbagba with mild resistance to black sigatoka (Nwauzoma *et al*., 2002) and off-type banana cv. Williams with resistance to yellow sigatoka disease (Trujillo and Garcia, 1996). Also reported are off-type banana plants cv. Grand nain with tolerance to panama disease (Roux, 2004), somatic embryo-derived off-type barley with tolerance to powdery mildew (Li *et al*., 2001) and off-type potato with tolerance to late blight (*Phytophthora infestans* (Mont.) de Bary) (Cassells *et al*., 1991). Black sigatoka is among the major diseases of banana with severe yield losses ranging from 30 to 70% (Tushemereirwe, 1987). The yield losses due to black sigatoka disease are ensued from the reduction in the number of fruits per bunch and fruit weight (Mobambo *et al*., 1993).

Micropropagated derived phenotypically normal plants exhibited higher susceptibility to black sigatoka disease than the CP derived plants. A reduced tolerance to fungal diseases has also been reported in many MP derived phenotypically normal plant species, including sugarcane, strawberries, African plantains and rhubarb line PC49 (Shoemark and Swartz, 1985; Péros *et al*., 1994; Vuylsteke, 1998; Zhao, 2004). The high susceptibility to fungal diseases of axenic *in vitro* regenerants has been associated with an alteration in leaf structural properties (Strange, 2003; Zhao, 2004) and a loss in plant natural defence due to removal of endophytic bacteria during tissue culture process (Hamill *et al*., 2005).

The off-type and MP derived banana plants manifested higher physiological injury as a result of water stress than the CP derived plants. The leaf senescence and death of the *in vitro* derived plants was probably due to higher water loss by transpiration as also reported in many field-grown micropropagated plant species (Marin *et al*., 1988; Kirdmanee *et al*., 1996; Herman, 2000). Water stress is among the major constraints resulting in substantial yield loss in rain fed-agriculture through a reduction in plant growth, fruit initiation and bunch weight (Robinson and Alberts, 1986; Saneoka *et al*., 2004). Currently, only banana groups with two B genomes such as Bluggoe (ABB) and Pisang Awak (ABB) are known to have significant natural tolerance to drought stress under tropical conditions (Simmonds, 1966; Thomas *et al*., 1998).

The off-type banana plants produced more suckers during water stress than the true-to-type plants. The enhanced sucker production is essential for plant survival during extensive drought stress. Earlier before this trial, it was noted that all field-grown off-type plant stools survived during the severe drought stress that hit the country in 2005, while 33% each of the MP and CP derived plant stools died. This survival mechanism possibly involves reallocation of water, nutrients and growth regulators towards the corm in the ground. The movement of nutrients, water and growth regulators from banana pseudostems towards corms after bunch harvest and their effect on the performance of next ratoon crop cycle has been documented. For instance, cutting banana pseudostems at 200 cm high above the ground after bunch harvest increases the bunch weight by 12% and reduces the time to harvest by 5% of the next ratoon crop in comparison with the removal of the whole pseudostems (Daniells and O'Farrell, 1987). More specifically, the earliness in sucker production in banana is promoted by a dense root system through its influence in cytokinin and gibberellic acid levels (Smith *et al*., 2001).

Cellular mechanisms underlying plant biotic and abiotic stress responses: The off-type banana plants had higher leaf stomata density, followed by the MP and CP derived plants. The observed drought stress susceptibility of the off-type and MP derived plants was probably due to higher water loss by transpiration caused by high stomatal density. A high leaf stomata density has also been reported in tissue culture derived plant species, namely bamboo, carnation and apple (Blanke and Belcher, 1989; Majada *et al*., 1998). The number and function of the stomata is determined at early stage of leaf development by environmental conditions, including humidity, temperatures, gaseous exchanges and liquid state of culture growth media (Jonathan *et al*., 1997; Majada *et al*., 1998). Carnation plantlets, *in vitro* propagated under humid condition using airtight culture vessels, had a higher stomata density, with some stomata functioning poorly when subjected to darkness, abscissic acid and polyethylene glycol (Majada *et al*., 1998).

The off-type banana plant leaves were more hydrophobic, which agreed with the gravimetrically determined leaf epicuticular wax content. The high leaf waxiness of the off-type plants agreed with their tolerance to black sigatoka disease. The water-dependant ascospores are the most abundant and infective structures of *Mycosphaerella fijiensis* (Stover and Simmonds, 1987; Stansbury *et al*., 2000). After the dispersal, the ascospores germinate on the moist lower leaf surface and the hyphae eventually emerge from a stoma where they either develop into conidiophores or grow across the surface to infect adjacent stomata (Stover, 1980; Stover and Simmonds, 1987). Leaf epicuticular waxiness through its influence on surface wettability has been associated with plant tolerance to fungal diseases (Gosowski, 1990; Strange, 2003). Conversely, the increased leaf waxiness of the off-type banana plants contradicted with its high water stress injury. The lack of positive correlation of leaf waxiness with drought stress tolerance has also been reported in some plant species, including maize (Ristic and Jenks, 2002). Leaf epicuticular wax deposition is influenced by environmental conditions and increases as *in vitro* plants acclimatise themselves to the *ex vitro* environment. The induction of wax deposition on leaves to protect plants against existing and subsequent water-limiting environments has also been reported in several plant species, including rose, cotton and sesame (Jenks and Ashworth, 1999; Jenks *et al*., 2001; Cameron *et al*., 2006; Kim *et al*., 2006; Koch *et al*., 2006).

The high electrolyte leakage of mannitol-dehydrated off-type plant leaves indicates membrane damage and thus a high risk of cell desiccation due to water stress. These data agreed with the observed drought stress susceptibility of the off-type plants. The high electrolyte leakage of the off-type plants positively correlated with the high antioxidant activity in the water-stressed cigar leaves. A high antioxidant activity is an indicator of increased oxidative stress and agreed well with the tolerance to black sigatoka disease of the off-type plants. The metabolites from *Mycosphaerella fijiensis* contain phytotoxins, which accelerate the oxidation of ascorbic acid (the most abundant antioxidant in leaf chloroplast) and induce necrotic lesions on infected banana leaves (Molina and Krausz, 1989; Busogoro *et al*., 2004). Thus, these authors hypothesised that the pathogenicity of black sigatoka pathogen involves the oxidative damage of leaf chloroplast. The increased antioxidant activity, however, contradicts with the water stress injury observed in the off-type plants. The lack of correlation of antioxidant activity with water-stress injury is probably due to the genetic/epigenetic difference between the off-type and true-to-type plants, which possibly affects background antioxidant levels. Antioxidants have positively been associated with plant resistance to environmental stress, including water deficit (Selote and Khanna-Chopra, 2004; Bor *et al*., 2003).

CONCLUSION

Off-type banana plants with increased tolerance to black sigatoka disease and plant suckering as a consequence of severe drought stress can be obtained from micropropagated landrace Uganda. This valuable variation is also associated with increased plant physiological injury as a result of drought stress. The tolerance to black sigatoka disease was possibly due to increased leaf epicuticular waxiness and total antioxidant activity, while the susceptibility to drought stress was due to high stomatal density and cell membrane instability. These findings suggest that somaclonal variation could be utilised as a tool for the improvement of plant tolerance to black sigatoka disease and increased plant suckering rate during severe water stress. The close relationship between water stress response and cell membrane injury suggests that mannitol-induced tissue dehydration might be used as a technique for early detection of off-type banana plants that are vulnerable to water stress.

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