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Impact of in vitro cold stress on two banana genotypes based on physio-biochemical Evaluation

M.T. El-Mahdy [a], M. Youssef [b], M.A. Eissa [c,*]

[a] Department of Pomology, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt
[b] Genetics Department, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt
[c] Department of Soils and Water, Faculty of Agriculture, Assiut University, Assiut 71526, Egypt

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ABSTRACT

Climatic changes affect agriculture and alter cultivated land area worldwide. Among which, low-temperature has a general negative impact on plant growth and productivity especially on tropical and subtropical species. To assess the effect of cold stress on banana, two global cultivated varieties (i.e. Grand Nain and Williams) were used for in vitro physio-biochemical evaluation. Cold stress was performed at constant 5 °C for 6, 12, 24 and 48 h, to assess the changes in the pool of proline, total phenolics, total soluble carbohydrates, K^+ and Ca^{+2} ions as well as the photosynthesis related pigments chlorophyll and carotenoids). Significant differences between cultivars and among exposure times were found in most of the studied traits. Results indicated that cold-stressed plants were capable to enhance their cold tolerance by over-accumulation of cryoprotectants, particularly under extended cold stress. Importantly, Williams showed more tolerance than Grand Nain by accumulating higher amounts of total phenolics, total soluble carbohydrates, higher concentrations of K^+ and Ca^{+2} ions as well as more content of the photosynthetic pigments, compared to Grand Nain. Meanwhile, Grand Nain produced greater amounts of proline than Williams. These findings suggest that compatible solutes increased in relation to cold tolerance mechanisms which modulate chilling-induced oxidative damage in banana. These imperative mechanisms could be utilized as important tools and selectable markers for cold tolerance screening in banana genotypes which could be helpful in breeding programs.

1. Introduction

Contemporary agricultural systems face various challenges, much more associated with changing climatic conditions. Recently, the occurrence of extreme meteorological events is increasing and is expected to threaten the plant life. Low temperature is one of the main constraints that profoundly hamper plant productivity and growth (Jha et al., 2017). Many areas of the world are affected by cold stress; about 64% of the earth's surface suffers from temperature dropping below zero (Źróbek-Sokolnik, 2012). Hence, it is very important to study the mechanisms underlying stress by which plants achieve cold tolerance.

Banana and Plantain (*Musa* spp., *Musaceae* family) are valuable fruit crops worldwide and currently grown in more than 130 countries. On the global scale, banana is ranking fourth in economic in economic importance after cereals with an annual production of more than 140 million tons (FAO, 2015). Plant species of tropical and subtropical origins including banana are highly susceptible to chilling (0–15 °C) and freezing (<0 °C) temperatures (Ravi and Vaganan, 2016; He et al., 2018). Every biochemical and physiological process has its response rate due to the minimum, maximum and optimum temperatures (Thakur et al., 2010). Thus, temperature triggers plant functions by affecting the growth and metabolism. The major cold stress symptoms include; growth reduction, leaf wilting, chlorosis (Cao et al., 2015), inhibition of photosynthesis performance, changes in membrane integrity and loss of cell compartmentation (Bo et al., 2017), impairment of enzymatic activity (Cheng and Song, 2006), miss-formation of protein synthesis (Mahajan and Tuteja, 2005) and over-accumulation of reactive oxygen species (ROS) which ultimately may cause plant death (Suzuki et al., 2012). Bananas are thermophiles, perennial herbaceous plants, grow and produce fruits all over the year, thus, in turn, the plant is frequently subjected to low temperature stress in winter which reduced yield significantly (Feng et al., 2015). Under sufficient water supplies, banana growth and yield are mainly determined by temperature (Robinson and Saúco, 2010). Moreover, moderate temperature (20–30 °C) is an essential requirement for appropriate vegetative growth, fruit development and maximum yield (Simmonds, 1962). However, temperatures lower than 20 °C negatively affect the phenological and ripening phases depending on cold intensity and its exposure time (Stover and Simmonds, 1987). Below 10 °C, leaf emergence stops with occurrence of malformations of bunches (Ravi and Mustaffa, 2013) and discoloration of peel tissues with abnormal fruit ripening (Chen et al., 2008). Moreover,

* Corresponding author.
E-mail address: mamdouh.eisa@aun.edu.eg (M.A. Eissa).

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irreversible chilling damage to the whole growth was reported in banana after exposure to 4 °C for four hours (Turner, 2003).

Plant species vary in their tolerance and susceptibility for cold stress based on their ability to withstand chilling injury and can be divided into tolerant and susceptible. The influence of cold stress on plants is complicated, under which several protective adaptations are expressed by plants. To cope with low-temperature stress, tolerant genotypes sense the fluctuation temperatures and reprogram their mechanisms via the induction of numerous biomolecules, this phenomenon is defined by cold acclimation (Theocharis et al., 2012). Modification of protective molecules which include antioxidants as phenolic compounds, amino acids such as proline, mineral nutrients, e. g., calcium and potassium, total soluble carbohydrates and maintenance of photosynthetic efficiency, are among these defense strategies which is widely reported in many plant species (Rooy et al., 2017; Janmohammadi et al., 2018).

It is important to screen for superior genotypes with a high degree of tolerance to chilling stress. Understanding the mechanisms and their regulation that control chilling stress response is essential in plant breeding programs. Although, many studies indicated the cold stress influence in Williams banana (Zhang et al., 2012); limited information is known about the susceptibility of Grand Nain cultivar to low-temperature stress. As Williams and Grand Nain are the most two important banana cultivars, they are extensively cultivated in Egypt and have an increasing economic concern for local market and export. Therefore, the present study was conducted to assess the effect of chilling stress in two banana cultivars (Williams and Grand Nain), at the physiological and biochemical levels in terms of chlorophyll, proline, carbohydrates, essential nutrients and phenolics contents under in vitro conditions to provide the possible markers toward improving tolerance of plant to low temperatures.

2. Materials and methods

2.1. Plant material and establishment for aseptic culture

Aseptic cultures of two cultivars of Cavendish banana (*Musa acuminata* Colla, AAA), i.e., Williams and Grand Nain were established in vitro as described by Strosse et al. (2003) with some modifications. All leaves and roots were removed from suckers followed by washing thoroughly under running tap water to get rid of remained soil. Explants were dipped in 100% (v/v) commercial bleach (5% sodium hypochlorite) for 25 min after minimizing their size to 10 × 2.5 cm. Thereafter, under aseptic conditions, explants with shoot apices were reduced in size to 4 × 1 cm and treated with 10% (v/v) commercial bleach for 10 min with gentle shaking and subsequently rinsed three times with sterilized distilled water. Lastly, sterilized explants were re-minimized to 1 × 0.5 cm then cultured (explant/jar) on 40 mL of full strength Murashige and Skoog (1962) (MS) medium fortified with 9.0 μM 6-benzyleaminopurine (BAP), 1.0 μM Indole-3-acetic acid (IAA), 1.25 mM KH_2PO_4, 3% (w/v) sucrose. Obtained shoots were subcultured every 30 days in the following MS multiplication medium: 22 μM BAP, 1.25 mM KH_2PO_4, 3% (w/v) sucrose. Gelrite (0.2% w/v) was used for solidification after adjusting the pH of all media to 5.8 then media was autoclaved. Cultures were maintained at 26 ± 1 °C under a 16/8-h photoperiod.

2.2. Chilling treatments

After two subcultures, well-developed shoots were selected for chilling stress treatments. Uniformed, 4 weeks-old plantlets of the two cultivars were incubated in a cold growth chamber at constant 5 °C under continuous fluorescent light for 6, 12, 24, and 48 h. Plants of the control were kept in a growth chamber at 25 °C under same light conditions. After treatment, samples were collected from control and chilling treated plants. The fresh and dry weights of plant samples were recorded and then the samples were kept for further analysis.

2.3. Photosynthetic pigments

The photosynthetic pigments; chlorophyll a (Chl-a), chlorophyll b (Chl-b), total chlorophyll (Chl a + b), their ratio (Chl a/Chl b) and carotenoid contents were assessed following the modified protocol of Lichtenthaler (1987). Briefly, 100 mg of fully expanded fresh leaf was extracted with 10 mL ethyl alcohol (95%) in a test tube and kept in darkness until the sample's color completely turned into white. Chl-a and Chl-b concentrations were measured by spectrophotometer at 663 and 644 nm, respectively. Carotenoid concentration was also determined spectrophotometrically using same plant extract at 470 nm. The blank was 95% ethyl alcohol.

2.4. Proline accumulation

Free proline level was measured in the fresh leaves according to the procedure of Bates et al. (1973) with little modifications. Plant extract was obtained from fresh leaf tissue (0.1 g) using 10 mL of 3% sulfosalicylic acid (w/v). Two milliliter of the extract were mixed with 2 mL of glacial acetic acid and 2 mL of acid–ninhydrin reagent in a test tube and incubated for 1 h at 100 °C. The reaction was then quickly arrested on ice before the addition of 4 mL of toluene followed by 30 s agitation on vortex. Samples were measured spectrophotometrically at 520 nm.

2.5. Total phenolics

Total phenolic content was estimated with a Folin–Ciocalteu assay (Ainsworth and Gillespie, 2007) with minor modifications. Samples of fresh leaves (0.1 g) were grounded in 10 mL methyl alcohol (85%) and subsequently centrifuged for 10 min at 6000 × *g*. 300 μL of plant extract was mixed with 1 mL of 10% Folin–Ciocalteu reagent. Then, after allowing the solution to react for about 5 min, 1 mL of 700 mM Na_2CO_3 was added to the mixture with agitation for 2 h at room temperature. The absorbance was recorded at 765 nm against gallic acid (blank) and expressed as mg/g FW.

2.6. Soluble carbohydrates

The anthrone colorimetric reagent was the basis to determine the total soluble carbohydrates content in the banana leaves (Yemm and Willis, 1954). Firstly, 5 mL of ethanol (80%) was added to 0.50 g fresh leaves for three times, then samples were centrifuged for 15 min at 1500 × *g*. Subsequently, 100 μL of the extract was added to 3 mL of 0.2% anthrone reagent. The solution mixture was boiled at 95 °C for 10 min and then, the reaction was terminated quickly in an ice bath. The absorbance of the samples was recorded spectrophotometrically at 620 nm using glucose as the standard and expressed as mg soluble carbohydrates g^{-1} fresh weight.

2.7. Mineral analysis

Total potassium (K) and calcium (Ca) concentrations were measured in plant samples by Inductivity Coupled Plasma Emission ICP 6200 (ICP) after wet digestion with a mixture of H_2O_2, Se, Li_2SO_4 and concentrated H_2SO_4, (Parkinson and Allen, 1975; Amin and Eissa, 2017). The concentrations of Ca and K were expressed as mg kg^{-1} of dry weights.

2.8. Data analysis

Chilling stress experiment was achieved in a complete randomized design with three replicates per treatment, each with four plantlets. A total of 120 plantlets were evaluated (2 cultivars × 5 treatments × 3 replicates × 4 plantlets). MSTAT-C C micro-program (Nissen, 1984) was