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EX-SITU CONSERVATION AND MORPHO-BIOCHEMICAL ANALYSIS OF EXOTIC CULTIVARS OF BANANA

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Abstract

Impact of various (growth/storage) media (SM1 to SM8) on biochemical and morphological traits of three cultivars of banana has been observed in this current study (Pisange, Brazillian and William). Study was carried out using two different temperature treatments *i.e.* 26°C and 18°C in order to develop an effective protocol for a short term *ex situ* conservation. Research experiments were carried out at National Agricultural Research Center (NARC), Islamabad, Pakistan during 2015. Maximum variations were observed for all of the morphological traits at a varied temperature and media concentrations. At 26°C, enhanced growth (plant height) and increased number of shoots had been observed in all cultivars, while the rate of increased number of roots was non-significant at both temperatures. After conserving the cultivars for short term of 5 months, biochemical analysis was performed. The biochemical analysis revealed the significant variations at both of the temperatures as well as on media. Brazilian cultivar (cultivar 2) substantially accumulated higher concentration of soluble sugar and proline both of provided temperatures as compared to other genotypes Cultivar 3 showed a significant increase in total chlorophyll content and chlorophyll a, b (William). The overall maximum proline and contents of chlorophyll were recorded in the cultures that were incubated at 26°C and 18°C respectively. So, 18°C temperature in combination with media SM2 and SM3 is better recommendation for short-term conservation of banana cultivars *ex situ* while 26°C is the best recommended temperature for maximum growth.

Key words: Banana, Conservation, Cultivar, Growth, Morpho-biochemical.

Introduction

Banana (*Musa* spp.) has primary origin of Asia and Africa and it belongs to an important monocot family *Musaceae* and order zingiberales (Stover & Simmonds, 1987). Banana is the most important and major food especially in tropical and sub-tropical regions and it is also the most widely exported fruit in the world. Approximately 95.6 million tons of bananas are produced in a year and are grown in about 150 countries all around the globe (Singh *et al.,* 2011). After rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mayz*), banana is the major and prominent crop food and is the one of the important commodities in an international trade and here it is ranked as the 4th largest food internationally (Singh *et al.,* 2011).

Both biotic and biotic factors affect the morpho-biochemical and physiological properties of plant (Jan *et al.,* 2016; Jan *et al.,* 2017). The crop of banana is at the risk of biotic stresses such as *Banana streak virus*, *Banana bract mosaic virus*, *Cucumber mosaic virus* and *Banana bunchy top virus* which are the four important and widely spread viral diseases. These diseases are disturbing the production rate of banana crop while the abiotic stresses such as the environmental factors also play role in minimizing banana's production specifically among the smaller scale farmers those have inadequate resources. Banana crop production is often put to an end usually because of natural disasters (Singh *et al.,* 2011).

Propagation of crops by using tissue culture technique (TC) is the simple and first solution of the problems of such rapidly evolving ailments (Abbasi *et al.,* 2011; Ahmad *et al.,* 2011; Ali *et al.,* 2011; Hussain *et al.,* 2013; Khan *et al.,* 2014; Jan *et al.,* 2015). The technology deals with the micro propagation and produce healthy planting germplasm. From old methods (usually sucker-propagation, the *In vitro* banana tissue culture technology is best and more considerate about banana production, consistency, ideal yield, healthy planting germplasm and true to type plants (Hwan *et al.,* 1976). For commercialization, it is important that good quality bananas are produced to keep in mind and fulfill the increased demand of constant supplies. Moreover TC methods allow rapid and efficient production of many important plant species (Engelmann, 1991; Jabeen *et al.,* 2017; Hussain *et al.,* 2018; Naz *et al.,* 2018; Kausar *et al.,* 2019). Though, the higher duplication rates are usually achieved with *In vitro* techniques which make possible continues production of massive amounts of plant material or germplasm. But, the continuous production of such massive amount of plant germplasm leads to create a trouble in the organization and management of such huge *In vitro* propagated collections of plantlets, and 2nd problem is, by doing continues culturing the somaclonal variations occur due to which the risks of losing genetic material increased. Contamination or anthropoid errors also increase the risks of losing the genetic integrity plantlets (Scowcroft, 1984).

The basic standard on the *In vitro* storing of genetic assets is to reduce speed of the plantlets growth or sprouting. The present study is totally based on this context, the experiment was designed to optimize temperature and storage media for banana's explants on the *In vitro* by (i) evaluating the influence of different

protocols of media on *In vitro* storing of banana (ii) optimizing storage temperature for short-term *In vitro* conservation of *Musa*germplasm (iii) physiological and biochemical analysis such as proline, protein, sugar and chlorophyll determination tests of banana for selection of suitable temperature and media for *In vitro* conservation of banana.

Materials and Methods

The present research was performed to bring out an effective protocol for the short-term *ex situ* conservation of banana exotic cultivars C1, C2 and C3 *i.e.,* (Pisange, Brazillian and William). Research was conducted at *In vitro* lab of Plant Genetic Resources Institute (PGRI), NARC, Islamabad, Pakistan. Physiological and Biochemical experiments were executed using the physiology and biochemistry lab of NARC Islamabad.

Experimental design and material of plant: The analysis was laid out by using data of three banana cultivars with three replicates in Complete Randomized Design (CRD). One month grown sword suckers of selected cultivars were used. Then the sprouts/shoots of these grown cultures from sword sucker were used for experimentation with two temperature treatments and eight different media (Table 1).

Culture initiation: For culture initiation, banana selected cultivars sucker taken from NARC fields Thatta (Sindh) were used. These selected cultivars were C1, C2 and C3 (Pisang, Brazilian and William). After trimming these suckers were surface. The sterilized suckers were then cultured on MS medium under sterilized condition MS medium was supplemented with 0.1 mg/l Indole Acetic Acid and 8 mg/l 6-Benzoyl Amino purine. The temperature kept in growth room for culture initiation 25±2°C for 16 hours photoperiod, illuminated with 2000 *lux* light intensity.

After one and half month of incubation the cultures were grown and shifted to multiplication medium. The multiplication medium recipe was MS basal salts (Murashige & Skoog, 1962) supplemented with different growth regulators and vitamins such as IAA 1ml/l, BAP6 ml/l, $KH_2PO_4$1ml/l thiamine HCL, 5 ml/l, sugar 30 g/l and gel1.8 g/l.

Banana cultivars conservation experiment

Treatments for slow growth: For conservation experiment various slow growth/storage media (SM1, SM2, SM3, SM4, SM5, SM6, SM7, and SM8) and 2 storing temperatures *i.e.,* 26°C and 18°C were used (Table 1).

Individual shoots were isolated from healthy multiple shoots cultures of *Musa sapientum* cultivars C1, C2 and C3 (Pisang, Brazilian and William) and single shoot was cultured in jars of all above eight storage media and then incubated at two diverse storage temperature *i.e.,* 18ºC and 26ºC. Three replicates of all the three cultivars at both temperatures and all conservation media were incubated. Culture was stored for periods of five months; data were noted on a regular basis each after a month interval.

Biochemical analysis: After short-term (5 months) conservation, the biochemical analyses were performed of all the stored cultures of banana cultivars C1, C2 and C3 (Pisang, Brazillian and William). These physiological analyses were carried out to recognize the variability due to different storage treatments in the biochemistry of conserved plantlets. The tests performed were Proline, Soluble Sugar and Chlorophyll contents determination tests. Proline content was estimated by using method of Bates *et al.,* (1973) while soluble sugar content of sprouts was estimated done by the techniques of Dubois *et al.,* (1956). Chlorophyll a, b and total chlorophyll contents were estimated according to Arnon (1949).

Statistical Analysis

The statistic software Statistix 8.1 was used for the interpretation of all the data collected during five months of research.

Table 1. Various slow growth/Storage media (SM) for *In vitro* conservation of three exotic cultivars of banana.

S. No.	Treatments	Storage temperatures
SM1	4.43 g/l (MS), 30 g/l (sucrose/sugar), 1.8 g/l (Gel). (Control)	26°C/ 18°C
SM2	4.43 g/l (MS), 2.25 mg/l (6-benzylo amino purine), 0.175 mg/l (Indole acetic acid), 100 mg/l (Myoinisitol), 2 mg/l (Glycine), 10 mg/l (Citric acid), 0.5 mg/l (Nicotinic acid), 0.1 mg/l (Thymine), 30 g/l (sucrose/sugar), 1.8 g/l (Gel).	26°C /18°C
SM3	2.21 g/l (½ MS), 2.25 mg/l (6-benzylo amino purine), 0.175 mg/l (Indole acetic acid), 100 mg/l (Myoinisitol), 2 mg/l (Glycine), 10 mg/l (Citric acid), 0.5 mg/l (Nicotinic acid), 0.1 mg/l (Thymine), 30 g/l (sucrose/sugar), 1.8 g/l (Gel).	26°C / 18°C
SM4	4.43 g/l (MS), 10 µ m/l (6-benzylo amino purine) 1 µ m/l (Indole acetic acid), 100 mg/l), 250 mg (Cefotoxime), (Citric acid30 g/l (sucrose/sugar), 1.8 g/l (Gel)	26°C / 18°C
SM5	2.21 g/l (½ MS), 60 g/l (sucrose/sugar), 1.8 g/l (Gel)	26°C / 18°C
SM6	4.43 g/l (MS), 60 g/l (sucrose/sugar), 1.8 g/l (Gel)	26°C /18°C
SM7	4.43 g/l (MS) 20 g/l (sucrose/sugar), 1.8 g/l (Gel).	26°C/ 18°C
SM8	4.43 g/l (MS), 20 g/l (sucrose/sugar) 30g/l (glucose) (liquid media)	26°C / 18°C

Results and Discussion

In the current study an effort has been made to establish *In vitro* optimized protocols for banana germplasm conservation (gene banks). Storage experiment was done for checking the performance of three exotic cultivars C1, C2and C3 (Pisange, Brazillian and William) during short-term *ex situ* maintenance while in addition to explored their biochemical and physiological performance after five months conservation. For conservation high survival percentage of plantlets is mandatory. The morphological data showed non-significant changes for selected cultivars C1, C2 and C3. While considering biochemical analysis, the three cultivars C1, C2 and C3 showed higher substantial variations at each incubation temperature.

Our results showed maximum variation at both the storing temperatures. On comparing the results on both temperatures for every culture, maximum number of shoots was recorded at higher temperature 26°C, on the other hand low temperature 18°C showed minimum number of shoots per culture (Fig. 1). Our results confirmed that shoot initiation is encourages by high temperature whereas shoot growth at low temperature is altered. Media SM2 showed higher number of shoots in all the cultures incubated at 18°C as compared to SM3, SM4, SM5, SM6, SM7 and SM8. Optimum concentration of macro and micro nutrients and vitamins present only in SM2 media which accelerate and stimulate shoot initiation and growth during storing course of period and this may be the reason for higher shoot formation in this media. These results are also in favor of the findings of Sachs (1965). He reported that in inner side of the meristem of plant lot of energy is needed for corpus cells to multiply, first corpus cell enlarged and then primary formation of leaf sheet primordial takes place to make the new tissue and this result support our findings because media MS2contain a lot of nutrients which fulfill the requirements of energy for shoot formation. Stange (1965) also describe that next to energy, sprouts initiation also requires plenty supply of micro, macro nutrients and essential vitamins which justify present results.

Considerably number of roots per culture showed non-significant variations at various media. While the number of roots per cultures showed significant results at both the temperatures low and high (18, 26°C) in all the cultivars C1,C2 and C3 (Fig. 2). Our results are not in line with findings of Fonnesbech (1974). Fonnesbech reported that by increasing incubation temperature root formation increase in cultures means that according to him high temperature encourage root initiation. However the interaction among both the treatments as nutrient media and temperature showed substantial differences. Our findings matched with the results of George *et al.,* (2013) which stated that in banana the roots induction interaction of nutrient media and temperature influence root initiation significantly. In media SM2 highest (25) roots were recorded while at media SM8 lowest numbers of roots in the cultures incubated at both temperatures were recorded. SM2 media is supplemented with low salt concentration (½ MS) due to which roots to which higher number of roots develops in the cultures in media SM2 as decline of salt concentration cease physiological reactions and do stimulates root development. According to Bonner & Addicott (1937), in the root meristem some important vitamins, nicotinic acid and thiamine have a great effect on cellular proliferation of pea plants. These conclusions justify our results as in recipe of SM2 media all these vitamins such as nicotinic acid, thiamine and others are in specific combination due to which root growth is maximum in SM2 media in comparison to SM3-SM8.

The significant results were observed at different temperature treatments regarding to average length of shoots in cultures. Our results showed that all treatments as media and temperature both have positive effect on average length of shoots in each culture. At both temperatures greater average length of shoots recorded considerably in cultivar C2 at SM7 and SM1 respectively but cultivar C3 maximum average length of shoots were noted at SM6 and SM7 at both storing temperatures (18°C and 26°C). In media SM3, SM4 and SM2 smaller average length was observed randomly at both storing temperature. Overall results showed higher/greater average length of shoots at temperature 26°C (Fig. 3). Different media and temperature treatments showed different growth results for all the three exotic cultivars C1, C2, C3 which might be due to the variations genetic makeup of cultivars. Engelmann (1991) also rescored similar results.

For conservation of plants in *ex-situ* high survival percentage is mandatory. Temperature and media in specific combination directly affect the survival ratio of banana exotic cultivars C1, C2, C3 (Pisange, Brazillian, William) in the conservation period of five (05) months. Low temperature *i.e.* 18°C stored plantlets showed maximum survival percentage (100%) in all cultivars C1,C2,C3, while least survival percentage (0%) was recorded in plantlets shaded under 26°C (Figs. 4-6, 7a and 7b). These results agree with the results of Banerjee & De Langhe (1985). Who observed high survival rate in the explants incubated at a low temperature. In the same way our results favors the conclusions of Bhat & Chandel, (1993). They explained that for medium-term conservation of *Musa* species, temperature 15°C is best one and this may be due to the reason that due to high temperatures root growth become altered which then further decrease shelf life of cultures.

Both media composition and temperature have direct affect on plants survival rate of all three banana genotypes. At SM2 and SM3, highest survival percentage of 100% was recorded in relation with lower temperature 18°C. Salisbury and Ross (1985) support our results. They observed that, application of nutrients in small amount distorted the metabolic reactions that in return disturb the plantlets growth.

The abrupt growth of all three cultivar go down in the survival percentage was recorded with highest growth at higher temperature 26°C on various storage media and might be because of the exhaustion of nutrients. George (2013) observed similar results in *Musa sapientum.* While that plantlets incubated/stored under low temperature 18°Cat media SM2 and SM3 remains fresh lush green and healthy throughout the experimentation time period. Slow plantlet growth at 18°C was might be because they utilize least nutrients for survival at first and hence the remaining nutrients remain available for longer time till 5th months of storing period.

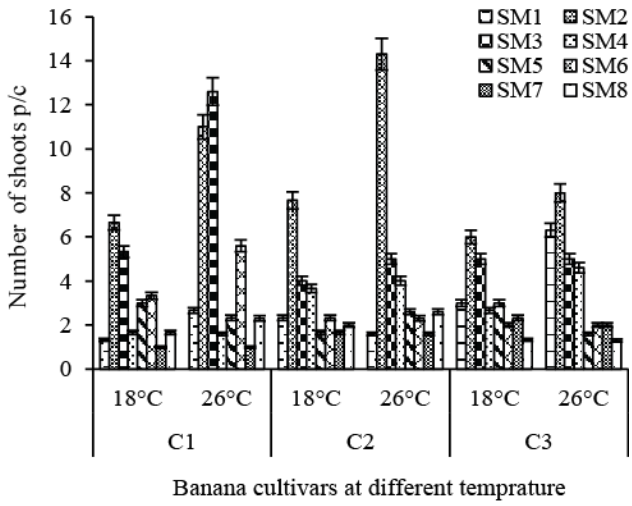

Fig. 1. Effect of temperature and media on number of shoots per culture of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

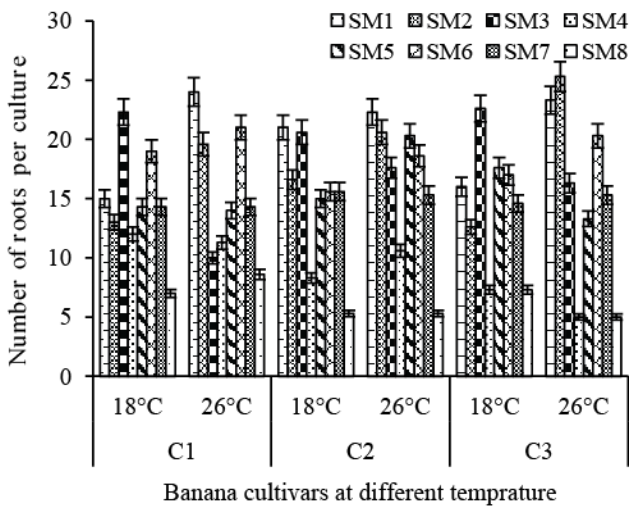

Fig. 2. Effect of temperature and media on number of roots per culture of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

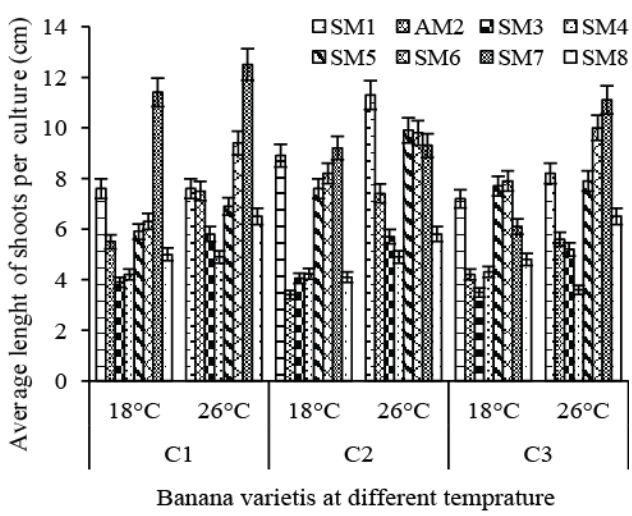

Fig. 3. Effect of temperature and media on average length of shoots per culture of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

Proline acts as osmo-sensors and important molecules for different stress recognition. In our results, substantially higher amount of proline content were observed in C2 (Brazillian) (Fig. 8). After 5 months of conservation higher consumption of Proline were recorded in all the cultivars at high temperature *i.e.* 26°C not in low temperature (18°C). Our results showed that indicated that plants undergoes in nutrient stress state due to exhaustion of nutrients and the plants consume proline content for avoiding stress condition. Jan *et al.,* (2016) reported that proline enhance plant growth under salt stress condition.

Lower variations for soluble sugar contents were observed at both storing temperatures *i.e.* 26°C and 18°C but all the media showed different effect in sugar contents on temperature treatment (Fig. 9). While a substantial variation in cultivar for sugar content level. Maximum accumulation of sugar content was determined in C2 (Brazillian). This experiment results showed that changes in sugar level might be due to the genetic variability of three cultivars. As sugar content in plants play a principal role as osmo-sensors and signaling molecules that regulate the metabolic system and plant growth (Arroyo *et al.,* 2003).

Higher chlorophyll accumulation indicates the good health of plants. Chlorophyll *a, b* and total chlorophyll contents were measured in plantlets after their short-term preservation. Significant variation at both the temperatures as well as different storage media for selected three (03) cultivars (Figs. 10-12). In plantlets stored at 18°C considerably high chlorophyll *a* content (0.2815) was observed. Kubota & Kozai (1994) results also support our results that optimized low temperature for conserving broccoli micro propagated sprouts as it was observed by an increase in chlorophyll amount in broccoli shoots.

In this study cultivar C3 (William) accumulate comparatively higher Chlorophyll *a, b* and total chlorophyll content and shows high tolerance toward different storing temperature. Highest chlorophyll *a, b* contents were noted in media SM2 and same results for total chlorophyll content were recorded in the cultures incubated at temperature 18°C. Plantlets of C1 and C3 but C2 showed maximum storage of chlorophyll content at SM1 and SM2 both which were non-significantly changed from each other. Higher temperature 26°C exhibited maximum chlorophyll *a* and total chlorophyll content at media SM1, SM2 and SM7 in cultivar C1, C2 and C3 randomly. These findings explained that at low temperature 18°C high accumulation of chlorophyll estimated at media SM2 which rise the percentage of plantlets survival as current results showed maximum survival proportion at SM2 (Figs. 10-12).

Conclusion

The 26°C is accurate and optimum temperature for maximum growth in 5 months storage period. While media SM2, SM3 and low temperature 18°C is most suitable for slow growth, maximum survival percentage (100%) with healthier, fresh plantlets for *ex situ* conservation of three banana exotic cultivars (C1-Pisange, C2-Brazillian, C3-William). Hence storage media SM2 and SM3 in combination with incubation temperature 18°C is suggested for short-term *ex situ* conservation of these exotic banana cultivars.

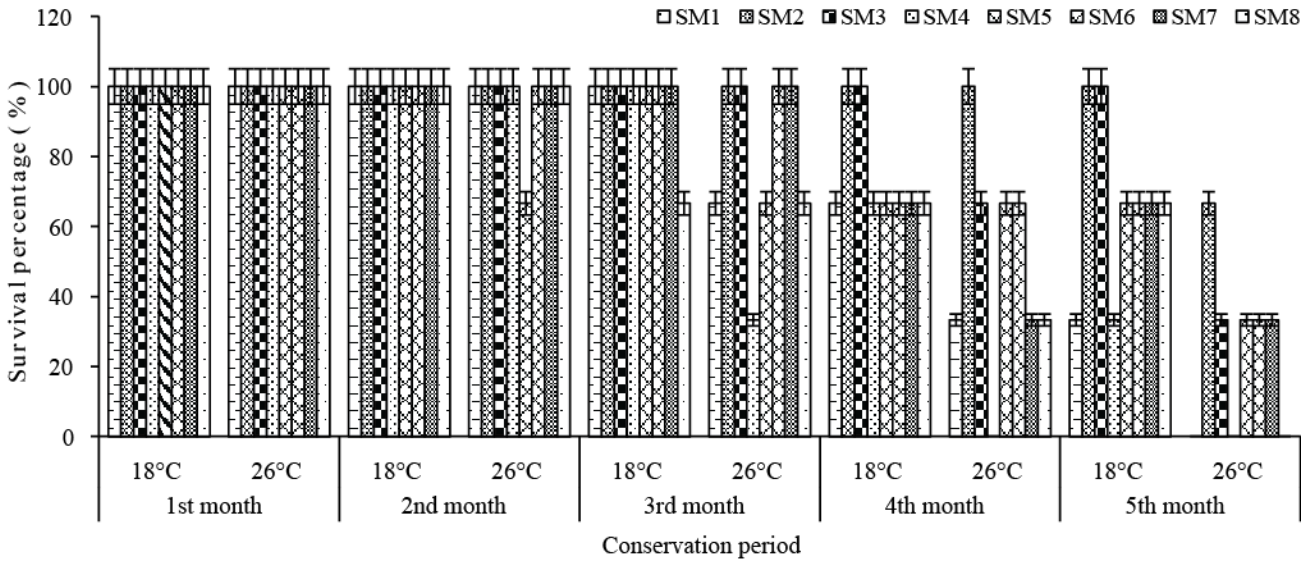

Fig. 4. Effect of temperature, media and incubation period on survival percentage of banana cultivar 1 (Pisange).

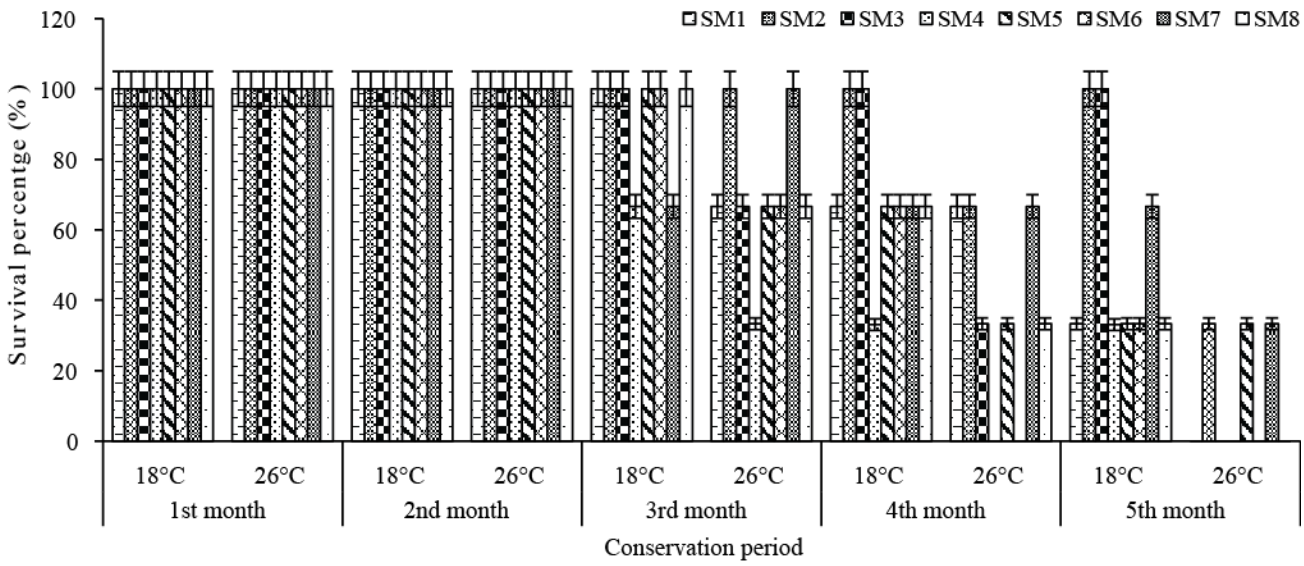

Fig. 5. Effect of temperature, media and incubation period on the survival percentage of banana cultivar 2 (Brazillian).

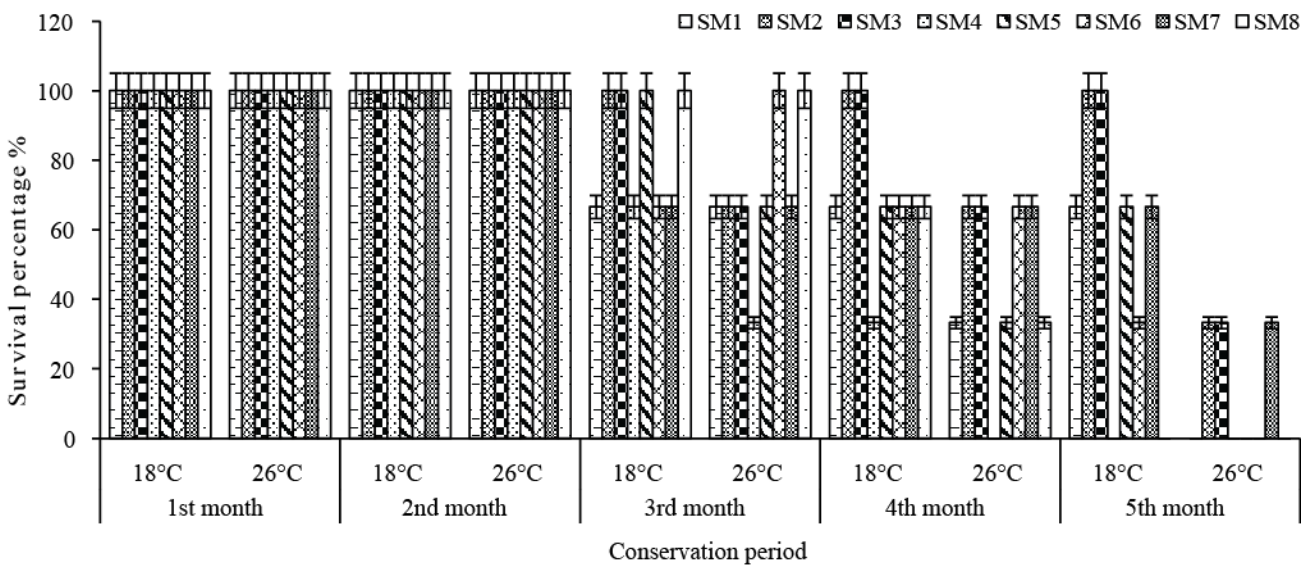

Fig. 6. Effect of temperature, media and incubation time on survival percentage of banana cultivar 3 (William).

Fig. 7(a). Plantlets survived for 5 months at media M3 and temperature 18°C of three different banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

Fig. 7(b). Plantlets survived for 5 months at media (SM2) and temperature 18°C of three different banana cultivars (C1-Pisange, C2-Brazillian and C3-William).

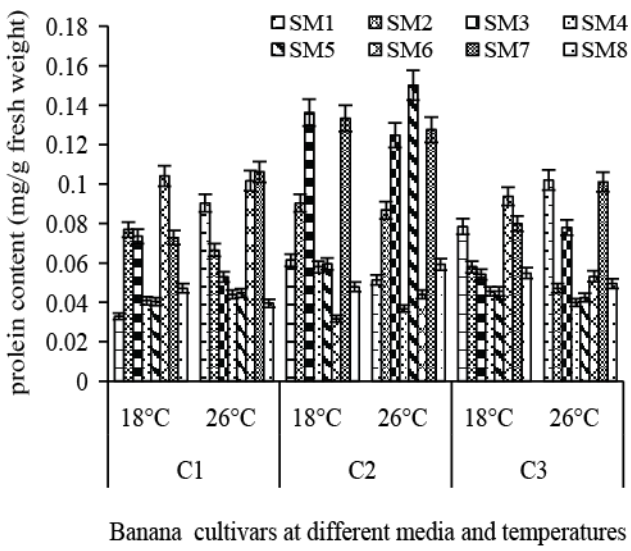

Fig. 8. Effect of temperature and media on proline content (mg g^{-1} fresh weight) of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

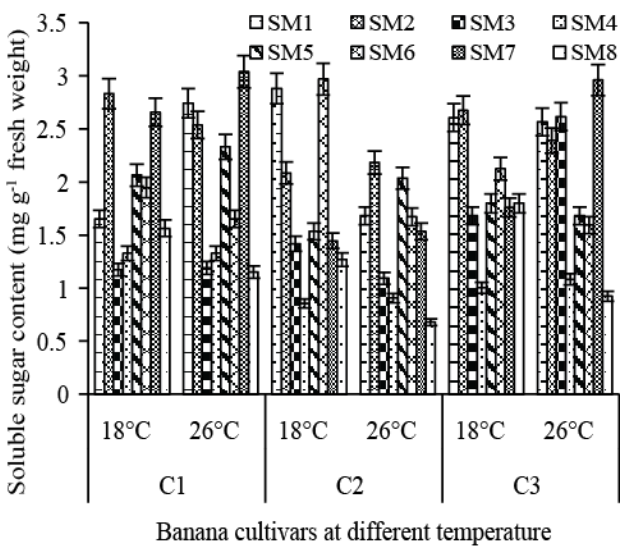

Fig. 9. Effect of temperature and media on soluble sugar content (mg g^{-1} fresh weight) of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

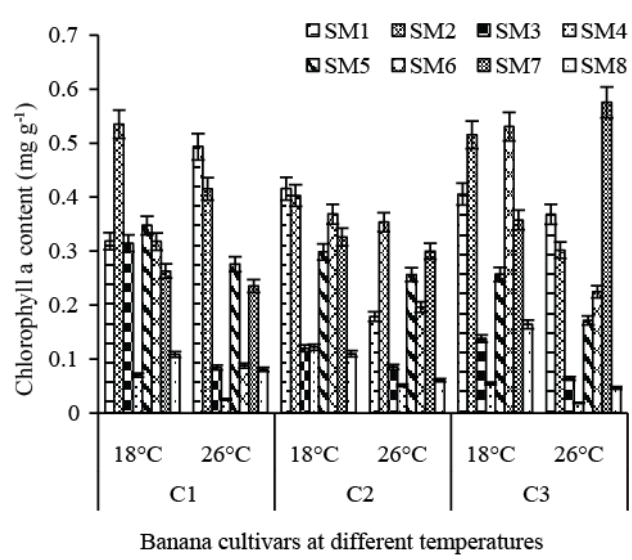

Fig. 10. Effect of temperature and media on chlorophyll *a* content (mg g^{-1} fresh weight) of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

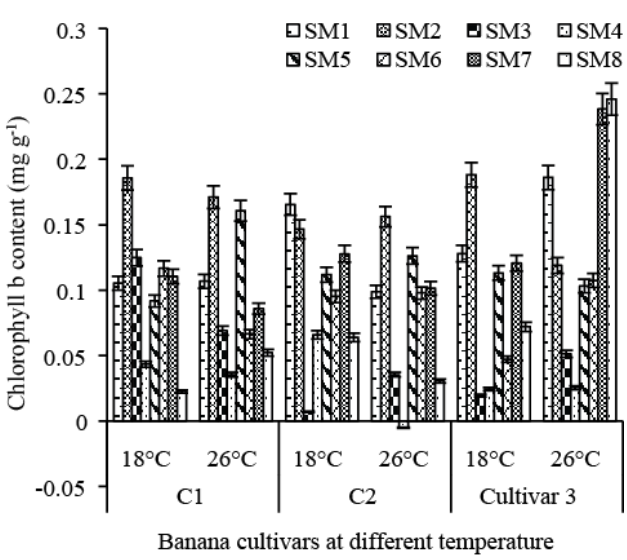

Fig. 11. Effect of temperature and media on number chlorophyll *b* content (mg g^{-1} fresh weight) of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

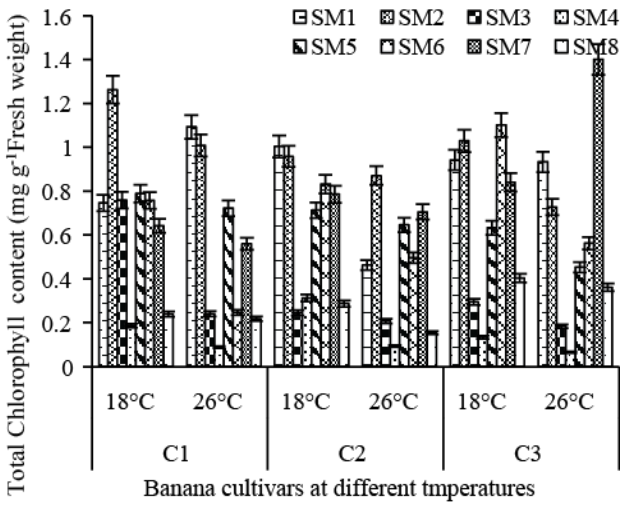

Fig. 12. Effect of temperature and media on total chlorophyll content (mg g^{-1} fresh weight) of three banana cultivars (C1-Pisange, C2-Brazillian, C3-William).

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