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Effects of chitosan on growth and physiological changes associated adaptation of *in vitro* obtained banana

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Abstract: Transfer banana plantlet from *in vitro* to *ex vitro* conditions is essential prerequisite in micropropagation. *In vitro* grown plantlets are unable to cope with soil environment. To avoid these challenges, the plant must be acclimatized according to a protocol that takes into account the economics of the plant concerned. Banana shoot culture was established on MS medium supplemented with 5 mg/l BAP and 30 g/l sucrose. Shoot cuttings were subjected for *in vitro* root formation on half strength MS medium containing 1 mg/l IBA. Plantlets were acclimatized under tissue culture room conditions where they were able to repair their physiological disorders and cope *ex vitro* conditions. Disorder repair processes were better if plantlets were treated with chitosan. When chitosan was used, efficiency of acclimatization increased during all acclimatization periods due to the increase in plant growth parameters, water content and ROS scavenging through the increase in activities of antioxidant enzymes such as superoxide dismutase and peroxidase. To save time and costs, root formation and acclimatization were combined in one step under transparent plastic bags (relative humidity 87%) in tissue culture room (25 ^{o}C) using *ex vitro*- hydroponic or soil-root formation procedure. Survival frequency was 100% when root formation was induced using 1 mg/l IBA and relative humidity was gradually decreased. Plantlets were transplanted in soil without application of *in vitro* or *ex vitro* root formation procedures died in five days.

Keywords: Acclimatization, antioxidant enzymes, banana, *Musa*, chitosan, growth, micropropagation.

Introduction

Since bananas (*Musa* spp.) are one of the most popular fruits all over the world, millions of people in several countries use bananas as a basic food source of great importance. Africa and Latin America are the largest banana-producing regions in the world, where 74.2% and 22.5% of the global production is produced, respectively (Rodriguez & Rodriguez, 2001). Bananas are rich in some minerals (phosphorus, calcium and potassium), vitamins (C, A, B1, B2 and U) and carbohydrates (Kodym & Zapata-Arias, 1999; Falcomer *et al*., 2019). Bananas contain potassium, which can help control hypertension (Zahra *et al*., 2021), however, it has also catecholamines that raise blood pressure if consumed in large quantities (Kuklin & Conger, 1995). For conventional banana propagation, corms, large and small suckers, and sward suckers are used (Arias, 1992) but these plant materials carry weevils, fungal pathogens, nematodes and viruses (Ganapathi *et al*., 2001). Therefore, plant tissue culture techniques are recommended to obtain multiplication in high quality, disease-free and uniform plants irrespective of the season (Hassanein, 2022).

Under *in vitro* culture conditions, regenerants are formed and grown under minimal abiotic stresses on a medium containing abundant nutrients and high humidity within culture vessel (Hassanein *et al*.,

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2022). Upon transferring of the in vitro obtained plantlets to ex vitro conditions, they expose to abiotic factors such as altered temperature, light intensity and humidity as well as biotic factors such as soil microflora. Therefore, micropropagated plantlets change their physiological and anatomical characteristics gradually following their transfer from culture container atmosphere to the natural environment that called acclimatization. Acclimatization may need several weeks to correct abnormalities in cuticle development, epicuticular waxes, stomata apparatus, photosynthetic pigments and chloroplast ultrastructure, abnormalities are previously formed under in vitro cultures (Pospíšilová *et al*., 2007).

Pospíšilová *et al*. (1999) demonstrated that during the first two weeks under ex vitro conditions, Chl a and Chl b contents as well as Chl a/b ratio were higher in tobacco plantlets grown under permeable closures than those grown under tightly closed glass vessels. This effect disappeared upon extension the exposure time (Pospíšilová *et al*., 1999). They also reported that stomatal conductance and stomatal transpiration rate decreased in three weeks but the cuticular transpiration rate decreased more slowly under ex vitro conditions. In addition, factors such as chitosan that reduce transpiration and maintain the valuable water content of the plant provide an opportunity to modify the abnormalities of the in vitro culture leading to successful acclimatization process (Veraplakom & Kudan, 2021; Kociecka & Liberack, 2021).

Chitosan can be used as antitranspirant and stimulate the physiological responses to mitigate the adverse effects of abiotic stresses through enhancement of antioxidant enzymes, photosynthetic rate and stomatal closure (O'Herlihy *et al*., 2003; El-Mougy *et al*., 2006; Hidangmayum *et al*., 2019). In addition, chitosan stimulates the production of osmotic adjustment compounds such as organic acids, sugars and amino acids (Hidangmayum *et al*., 2019) leading to improve plant health and yields (Metwaly *et al*., 2023). Since chitosan can inhibit the growth of different pathogens (Vasyukova *et al*., 2001), it can be used as alternative to chemical fungicides (O'Herlihy *et al*., 2003). Furthermore, irrigation with chitosan caused reduction of root-knot nematodes and Pochonia chlamydosporia infection (Escudero *et al*., 2017). Application of chitosan during micropropagation improves plantlet quality and facilitates acclimatization of plantlets to ex vitro conditions (Nge *et al*., 2006; Veraplakorn & Kudan, 2021). Chitosan can be used as bio-fertilizer with less impact on environmental contamination due to its biodegradability and non-toxicty (O'Herlihy *et al*., 2003; Abd-Elrahman *et al*., 2023). Pretreatment of different plant species with chitosan decreased lipid peroxidation, scavenge reactive oxygen species (ROS) and increase membrane stability (Bistgani *et al*., 2017; Hidangmayum *et al*., 2019). The ability of chitosan to enhance superoxide dismutase to scavenge superoxide anion was reported (Yang *et al*., 2009).

Insufficient acclimatization is the major limitation that retards large scale application of micropropagation technology due to high mortality (Deb & Imchen, 2010) leading to reduce the economics of tissue culture techniques in agriculture. To avoid wilting of plants after transplantation under ex vitro conditions, many attempts were made to decrease air humidity within jars such as increasing CO2 concentration via forced ventilation (Pospíšilová *et al*., 1999; Pospíšilová *et al*., 2007; Elisama *et al*., 2013) however, these attempts require sophisticated techniques and increase the cost of tissue culture products. Consequently, the aim of this work was to find alternative procedures to decrease plantlets mortality and shorten the acclimatization periods. In addition, this study enhances our limited understanding on the physiological changes accompanying plant acclimatization, in particular under the influence of some chemical compounds that control water loss, such as chitosan.

Materials and methods

Plant material and culture establishment

Suckers of field grown banana (Grand naine cultivar) were obtained from Sohag Governorate, Egypt and used as a source of shoot tip explants according to Hassanein *et al*. (2005). The obtained-sterilized shoot tips were cultured in glass jars contained about 25 ml of solid MS (Murashige & Skoog, 1962) medium supplemented with 5 mg/L benzyl amino purine (BAP). Initial banana explants were incubated in tissue culture room at 25±2 ºC, 16 h photoperiod with 100 µmol m-2s-1 and relative humidity of conditions (70%) for two months. During that time, cultured explants were aseptically transferred to fresh media three times at two weeks intervals. Then, in vitro obtained shoot tips were subcultured to obtain enough material for the following experiments.

In vitro root formation and acclimatization

For *in vitro* root formation, shoot cuttings were cultured on half strength MS medium supplemented with 1 mg/l indol 3-butyric acid (IBA) and 15 g/L sucrose for three weeks (Hassanein *et al*., 2003; Salem, 2020). Plantlets with well-developed roots

were washed under running tap water to remove agar residue.

Sixty plantlets (ten plantlets per timespan) were directly transferred into small pots containing a mixture of peat moss and sand (2:1 v/v) under transparent plastic bags for 0, 3, 7, 10, 14 or 21 days and incubated under tissue culture room conditions (27±2 °C, 16 h photoperiod with 100 µmol m-2s-1 and relative humidity of 70%). Sixty other plantlets (ten plantlets per timespan) were immersed in 0.1 % (w/v) chitosan solution for 5 minutes, transplanted into plastic pots containing soil mixture and covered with transparent plastic bags (relative humidity was 87%; measured by HANNA instruments - HI9065) under tissue culture room conditions for 0, 3, 7, 10, 14 or 21 days. Upon removal of the plastic bag covering the plants of the second group after any period of time, the plants were sprayed with 0.1% (w/v) chitosan solution once a time till the end of experiment. On the other hand, sixty plantlets were transplanted without covering the pots with plastic bags, and considered as control.

Since the fifth day, one-centimeter diameter pore was made in each bag and its diameter was doubled every five days for gradual reduction in relative humidity. Plantlets were watered each 3 days using tap water. Measurements were taken immediately after each acclimatization period, 0, 3, 7, 10, 14 or 21 days, and after 65 days. Experiment was repeated three times. Plantlets survival, length of shoot (cm), number of leaves and roots/ plantlet, fresh and dry mass/ plantlet, water soluble protein, water soluble carbohydrates, total amino acids, peroxidase (POX) and superoxide dismutase (SOD) activities, and POX patterns were evaluated.

Ex vitro root formation and acclimatization

For ex vitro induction of root formation (ex vitro hydroponic root formation procedure), fifteen shoots/treatment were soaked in 1, 2 or 3 mg/l IBA for 24 hours and transplanted into plastic pots (5 cm diameter) contained a mixture of peat moss and sand (2:1 v/v). Another fifteen shoots/treatment were directly transplanted into plastic puts containing a mixture of peat moss and sand (2:1 v/v) and watered by 1, 2 or 3 mg/l IBA (ex vitro soil root formation procedure). All plants were covered with plastic bags, watered with tap water/3 days and acclimatized as previously described. In addition, for each group, fifteen shoots were directly transferred (without acclimatization) to soil under tissue culture room condition (control). Shoot frequency, shoot length, root length, number of roots/plantlet and number of leaves/ plantlet, fresh and dry weights and water content were recorded.

Estimation of water-soluble carbohydrates

In test tubes, dry tissue of banana shoots (DW= 100 mg) was boiled in 10 ml of distilled H2O in water bath at 100°C for 2 h. After centrifugation, the obtained hydrolysate was completed to 10 ml using distilled H_2O. The anthrone sulphoric acid method that was reported by Fales (1951) and adapted by Grandya et al. (2000) was used.

Estimation of water-soluble protein

Powder of banana shoot tissue samples (DW= 50 mg) was boiled in 10 ml distilled water for two hours. The mixture was cooled and centrifuged. The volumes of the supernatants were standardized, transferred into clean tube and completed to 10 ml using distilled water. Water-soluble protein was determined as described by Lowery *et al*. (1951).

Estimation of total free amino acids

To determine the total free amino acid content of dry banana tissues, the ninhydrin test according to the method of Jones *et al*. (2002) was applied.

Estimation of photosynthetic pigments

To measure the photosynthetic pigment, banana samples were frozen in liquid nitrogen and saved under low temperature (-80 ℃). The photosynthetic pigments were determined according to Lichtenthaler (1987). In this procedure, 0.25 g of fresh tissue was extracted by using 5 ml of 80% (v/v) acetone. The extract was centrifuged at 11000 g for 10 min. Then, the extract was measured using spectrophotometer at the wavelengths of 646.8 and 663.2 nanometer to determine chlorophyll a (Chl.a) and b (Chl.b), respectively. The amount of pigment was calculated as described by Lichtenthaler (1987).

Estimation of peroxidase (POX) and superoxide dismutase (SOD) activities

For determination of peroxidase (POX) and superoxide dismutase (SOD) activities, protein content of 0.5 g leaf tissue was homogenized in 50 mM phosphate buffer (pH = 7) and 1% (w/v) polyvinylpyrrolidone. Mixtures were centrifuged at 15,000 g for 10 min at 4 °C. Supernatant was used to determine POX activity according to MacAdam *et al*. (1992) and SOD activity according to the method described by Kwon *et al*. (2002).

Peroxidase pattern

For analysis of peroxidase pattern, one gram of banana shoots was ground on ice in a mortar using 1 ml of 0.04 M Tris-HCl, pH 7.0, containing 0.002 M cysteine. The homogenate was centrifuged at 15000 g at 4ºC for 15 min. Then, native PAGE was performed using 7.5 % acrylamide slab gels. Gels were run at 10 mA per gel for 6 h at 4 ºC with 0.025 M Tris-HCl + 0.192 M glycine buffer, pH 8.9. The peroxidase bands

were stained by phosphate buffer, Guaiacol and H_2O_2 as described by Siegel & Galston (1967).

Statistical analysis

Experiments were completely designed in a randomized manner and data were calculated using standard deviation (SD) according to the method described by Snedecor & Cochran (1980). Additionally, one way analysis of variance (ANOVA) using SPSS 16 was used to analyze the data. The significance level was measured using the Tukey test at $p < 0.05$.

Results

Banana shoot culture was established by extraction of cone-shaped shoot tip explants from sword suckers and culturing on MS medium supplemented with 5 mg/l BAP and 30 g/l sucrose. Phenol accumulation on the initial explantswas avoided by aseptically transferring of cultured explants to fresh media (MS medium supplemented with 5 mg/l BAP and 30 g/l sucrose) three times at two weeks intervals. The obtained shoots on initial explant were subcultured on the same type of medium (MS medium supplemented with 5 mg/l BAP and 30 g/l sucrose), each three weeks to achieve mass multiplication (Figgure.1). In vitro obtained shoots were cut and used for determination of growth parameters under the applied conditions and induction of *in vitro* and *ex vitro* root formation.

Figgure. 1: *In vitro* shoots obtained from banana tissue culture with MS medium supplemented with 3 mg/ l BAP for six weeks.

Frequency of root formation

Ex vitro induction of root formation was carried out according to ex vitro hydroponic root formation procedure (Table 1 & Figgure 2) or ex vitro soil root formation procedure (Table 2). In these two applied procedures, root formation and acclimatization were combined in one stage. Frequency of root formation was increased significantly (94%) when hydroponic-ex vitro root induction was carried out using 2 mg/l IBA (Table 1).

When induction of root formation was directly induced according to soil-ex vitro procedure (Table 2), root formation frequency was also significantly increased using 2 mg/l IBA (80%), but it was lower than that of ex vitro hydroponic procedure. When the concentration of IBA was increased to 3 mg/l, the frequency of root formation of ex vitro soil procedure (70%) was higher in comparison with the hydroponic one (56.67%).

In vitro root formation procedure was carried out on half strength MS medium containing 1 mg/l IBA and 15 g/l sucrose for 3 weeks. Under these conditions, all shoot cuttings showed extensive root formation that was necessary to carry out acclimatization procedures.

Figgure 2: Shoot cuttings subjected to 0.1 mg/l IBA for 24 hours in closed jar to induce root formation (*ex vitro* hydroponic procedure).

Figgure. 3: Photograph shows ex vitro root formation on shoot cuttings using 0, 1, 2 mg/ L IBA (from right to left) and watered with tap water for six weeks.

Root numbers

When *ex vitro* root formation was induced either in a hydroponic environment (Table 1) or ex vitro-soil procedure (Table 2), the highest root number was obtained when 1 mg/l IBA was used. However, the number of roots decreased signeficantly with the increase of IBA concentration in both cases. Under the influence of IBA, the number of roots formed using ex vitro soil root formation procedure (Table.2) was higher than that formed using ex vitro hydroponic root formation procedure (Table 1). On the other hand, root length decreased significantly with the increase of IBA concentration in both culture condition (Tables 1 and 2, Fig. 3). However, shoot lengths of plantlets that formed using the ex vitro soil root formation procedure (Table 2) were better than others formed using ex vitro hydroponic root formation procedure (Table 1).

Table 1: Banana shoots were hydroponically subjected to different IBA concentrations for *ex vitro* root formation and acclimatized for three weeks under transparent plastic bags. Values are means of three replicates ± standard deviation (SD).

IBA concentration (mg/l)	Frequency %	Shoot length (cm)	root length (cm)	No. of roots/ plantlet	No. of leaves/ plantlet	F.w./ plantlet (g)	D.w./ plantlet (g)	Water content (%)
1	75.33±5.78	10.5±0.5	5.47±0.76	4.8±0	7±1	1.33±0.01	0.06±0.001	93.64±0.13
2	94±5.28*	9.2±0.1*	3.33±0.76*	2.33±0.58*	6.33±1.53	0.89±0.01*	0.05±0.007*	94.38±0.15
3	56.67±5.77*	6.8 ±0.5*	1.73 ± 0.25*	1.67±0.58*	4.33±0.58	0.48±0.02*	0.03±0.001*	92.97±0.04

* Means that differences were significant at $p < 0.05$, compared to control (1 mg/l IBA).

Table 2: Banana shoots were directly transplanted in soil mixture wetted by different concentrations of IBA for *ex vitro* root formation and acclimatized for three weeks under transparent plastic bags. Values are means of three replicates ± standard deviation (SD).

IBA con. (mg/l)	Frequency (%)	Shoot length (cm)	root length (cm)	No. of roots/ plantlet	No. of leaves/ plantlet	F.w./ plantlet (g)	D.w./ plantlet (g)	Water content (%)
1	76.67±5.78	10.0±0.5	5.17±0.76	4±0	6±1	1.21±0.01	0.06±0.001	94.94±0.13
2	80±10*	9.1±0.5*	3.2±0.1*	4±1	5.33±0.58	0.86±0.01*	0.04±0.001*	95.21±0.09
3	70±10*	8.2±0.76*	1.8±0.1*	3±1*	5±0	0.57±0.01*	0.04±0.001*	93.68±0.06

* Means that differences were significant at $p < 0.05$, compared to control (1 mg/l IBA).

Table 3: Banana shoots subjected for *in vitro* root formation on half strength MS supplemented with 1 mg/l IBA and 15 g /l sucrose for three weeks, and acclimatized for different periods with or without chitosan. Values are means of three replicates ± standard deviation (SD).

Acclimat. period (day)	Shoot length (cm)	No. of roots/plantlets	No. of leaves/ plantlets	F.w./ plantlets (g)	D.w./ plantlets (g)	Water content (%)
Without chitosan						
0	7 ± 1	4 ± 1	4.3 ± 0.6	0.95± 0.01	0.09 ± 0.02	92.30 ± 1.62
3	8 ± 1	4.7 ± 0.6	4.7 ± 0.6	1 ± 0.09	0.1 ± 0.01*	92.00 ± 0.71
7	8.5 ± 0.5	5.3 ± 0.58	6.7 ± 1.2	1 ± 0.1	0.1 ± 0.02*	90.00 ± 2.64
10	9.43 ± 0.12	5.7 ± 1.2	6.7 ± 1.5	1.3 ± 0.7	0.13 ± 0.07*	90.14 ± 1.33
14	9 ± 1.3	8.7 ± 1.5*	6.7 ± 0.6	1.4 ± 0.3	0.12 ± 0.03*	91.42 ± 1.71
21	14 ± 1.73*	8.7 ± 3*	6.7 ± 1.2	4 ± 0.8*	0.3 ± 0.05*	92.5 ± 1.81
With 0.1% chitosan						
0	8 ± 1	4.7 ± 2	6 ± 1	1.1 ± 0.12	0.1 ± 0.01	91.71 ± 0.27
3	9.3 ± 0.6	5 ± 2	6 ± 1	1.2 ± 0.1	0.1 ± 0.01	91.60 ± 1.74
7	10.3 ± 2	5 ± 1	6.7 ± 0.6	1.2 ± 0.12	0.12 ± 0.03	90 ± 1.73
10	10.3 ± 0.6	6.3 ± 1.2	**7 ± 0**	1.45 ± 0.35	0.14 ± 0.04	90.34 ± 0.55
14	10.7 ± 0.6	8.3 ± 3.2*	7 ± 0	1.7 ± 0.6	0.14 ± 0.08	91.8 ± 1.38
21	14.7 ± 1.2*	8 ± 0*	7.7 ± 0.6	4.65 ± 0.8*	0.3 ± 0.05*	93.79 ± 0.07

* Means that differences were significant at $p < 0.05$, compared to control (0 day).

Table 4: Banana shoots subjected for *in vitro* root formation on half strength MS supplemented with 1 mg/l IBA and 15 g/l sucrose for three weeks, acclimatized for different periods with or without chitosan and continued growth for 65 days; the last 44 days were in the open field. Values are means of three replicates ± standard deviation (SD).

Acclimat. period (day)	Shoot length (cm)	No. of roots/ plantlet	Root length (cm)	No. of leaves/ plantlet	F.w./ plantlet (g)	D.w./ plantlet (g)	Water content
Without chitosan							
0	12.7 ± 0.3	7.7 ± 2.5	11.8 ± 2.8	7 ± 1	2.5 ± 0.4	0.17 ± 0.03	93.12 ± 1.48
3	15.7 ± 0.8	7.8 ± 3.7	11.8 ± 2.8	7.3 ± 1.5	2.9 ± 0.1	0.3 ± 0.23	90.00 ± 8.36
7	12.7 ± 2.5	8± 0	8 ± 1.5	7.33 ± 0.6	3.1 ± 0.2	0.31 ± 0.1	90.12 ± 1.63
10	16.2 ± 0.3	9 ± 1.7	15.3 ± 1.2	8 ± 0	5.5 ± 0.7*	0.45 ± 0.06*	91.80 ± 0.37
14	17.2 ± 2.3*	9.3 ± 1	13.8 ± 2.5	8 ± 0	5.2 ± 2.7*	0.45 ± 0.3*	91.3 ± 1.48
21	14 ± 1	8.0 ± 0	11.3± 3	7 ± 0	3.2 ± 0.8	0.27 ± 0.09	91.56 ± 1.42
With 0.1% chitosan							
0	10 ± 0	9 ± 1.6	3 ± 0	6.3 ± 0.6	1.3 ± 0.4	0.1 ± 0.04	92.50 ± 1.60
3	17.3 ± 1*	14.2 ± 1*	9.7 ± 2*	7.3 ± 0.6	4.8 ± 0.3*	0.39 ± 0.03*	91.80 ± 0.6
7	15 ± 0.7*	12.5 ± 0.5	9.3 ± 1*	7.7 ± 0.6*	3.8 ± 0.5*	0.28 ± 0.01*	92.52 ± 0.65
10	17 ± 1*	19.6 ±1*	10 ± 1*	7.7± 0.6 *	5.0 ± 0.4*	0.39 ± 0.3*	92.2 ± 0.17
14	17.3 ± 2*	15.7 ± 5*	9.3 ± 1.5*	8.3 ± 0.6*	6.2 ± 0.2*	0.46 ± 0.02*	92.6 ± 0.13
21	15 ± 1*	11.7± 3	8.3 ± 0.6*	7 ± 0	4.5 ± 0.8*	0.35 ± 0.09*	92.22 ± 1.42

* Means that differences were significant at $p < 0.05$, compared to control (0 day).

When root formation was carried out *in vitro* on half strength MS medium with 1 mg/l IBA, each plantlet formed 4 or 5 roots with length of about 3-9 cm within 21 days. Furthermore, when rooted plantlets were transferred to acclimatize under transparent plant bags, the number of roots increased with the increasing of the acclimatization time up to 21 days (Table 3). In 65 days, the number of roots of different treatments did not differ significantly, while roots length increased non-significantly, with the appearance of secondary roots (Table 4 and Fig. 4). Data indicated that the number of roots during the acclimatization period was not affected by chitosan treatment (Tables 3 and 4).

Figgure 4: Shoot cuttings subjected to 0.1 mg/l IBA for 24 hours in closed jar to induce root formation (*ex vitro* hydroponic procedure).

In vitro or *ex vitro* root formation made the plantlets able to survive and grow under the conditions of the tissue culture room, where the temperature was 27 ^{o}C and the relative humidity was 87% (under plastic bag). On the contrary, plantlets were transferred without application of *in vitro* or *ex vitro* root formation procedures died within five days.

Growth and physiological parameters as influenced by chitosan treatment during *in vitro* acclimatization

Under *ex vitro* root formation and acclimatization for 21 days, plantlets showed different values of growth parameters and water contents. Application of 1 mg/l IBA during *ex vitro* hydroponic root formation procedure (Table 1) or soil root formation procedure (Table 2) resulted in the highest values of growth parameters. Plantlets water content of plants subjected for induction of root formation according to *ex vitro* soil procedure were higher than those of *ex vitro* hydroponic procedure.

Plants with *in vitro* formed roots showed a segnificant increase in the growth values with the increase of acclimatization period, in comparison to plantlets at zero day of acclimatization (Table 3 and 4). Also, the values of the aforementioned factors were higher in the chitosan-treated plants compared to the untreated ones. Under acclimatization of *in vitro* obtained plantlets and during the first 10 days, plantlets showed non-significant increase in growth parameters in comparison to plantlets at zero day of acclimatization. It is worth noting that the plants grown under the conditions of tissue cultures showed non-significant increase in water content, but it decreased immediately and non-significantly after transfer the plants from *in vitro* to *ex vitro* environment, regardless of the method of transplantation. The water content of plants treated with chitosan revealed non-significant increase higher than that of untreated plants (Tables 3 and 4). Under all applied acclimatization procedures, the water content raised again non-significantly within three weeks compared to that at zero day of acclimatization (Tables 3 and 4). Comparing the data recorded in Tables 1 and 2 with the data recorded in Tables 3 and 4 indicated that plants passed the acclimatization stage continued growing under open environment conditions.

The concentration of each pigment component or total pigment content decreased significantly (compared to those estimated at zero day of acclimatization) once the plants were transferred from the *in vitro* to *ex vitro* conditions during plantlets acclimatization, but their concentrations resumed increasing within 21 days (Tables 5). Moreover, the pigment content of plants treated with chitosan was higher than their peers that were not treated with it.

When plantlets were transplanted under *ex vitro* environment for acclimatization, amino acids decreased significantly in comparison to the control (plantlets obtained from *in vitro* conditions), the lowest value (1.68 mg/g DW) was recorded for plants that were exposed to acclimatization conditions for a period of 21 days (Table 6). The plant contents of soluble carbohydrates and soluble proteins decreased significantly after transferring the plantlets to the *ex vitro* environment in comparison to the control plants (*in vitro* obtained plantlets). The lowest value of soluble carbohydrates was recorded for plants that exposed to acclimatization conditions for a period of 7 days. By increasing the acclimatization period more than 7 days, plant soluble carbohydrates increased. While, the highest values of amino acids and soluble proteins were registered for *in vitro* grown plants (0 period; *in vitro* obtained plantlets), the highest value of soluble carbohydrates was registered when plantlets were acclimatized for 21 days. Treating plants with chitosan during the acclimatization periods did not change the recorded trend, but all values of amino acids, soluble carbohydrates and soluble proteins were higher in plants treated with chitosan than untreated plants.

Table 5: Pigments contents of banana plantlets under the influence of several acclimatization periods with or without chitosan treatment.

Treatments (days)	Chl. a (mg/g FW)	Chl. b (mg/g FW)	Chl. a/b (mg/g FW)	Carotenoid (mg/g FW)	Total pigments (mg/g FW)
Without chitosan					
0	0.67 ± 0.044	0.3 ± 0.02	2.3 ± 0.19	0.35 ± 0.035	1.32 ± 0.13
3	0.45 ± 0.044*	0.21 ± 0.02*	2.1 ± 0.16	0.36 ± 0.036	1.2 ± 0.17
7	0.43 ± 0.2*	0.21 ± 0.04	2.05 ± 2.36*	0.34 ± 0.01	1.0 ± 0.04*
10	0.14 ± 0.02*	0.87 ± 0.084*	1.6 ± 0.15	0.55 ± 0.015*	0.78 ± 0.12*
14	0.24 ± 0.026*	0.17 ± 0.001*	1.4 ± 0.08	0.5 ± 0.02*	0.91 ± 0.09
21	0.27 ± 0.05*	0.2 ± 0.08*	1.35 ± 0.07	0.6 ± 0.02*	1.07 ± 0.5*
With 0.1% chitosan					
0	0.61 ± 0.02	0.32 ± 0.02	1.9 ± 0.06	0.3 ± 0.01	1.23 ± 0.06
3	0.5 ± 0.04	0.24 ± 0.08*	2.01 ± 0.15	0.3 ± 0.02*	1.04 ± 0.01*
7	0.2 ± 0.03*	0.27 ± 0.06	0.74 ± 0.63	0.4 ± 0.01*	0.87 ± 0.01
10	0.11 ± 0.09*	0.53 ± 0.13*	0.21 ± 1	0.5 ± 0.02*	1.14 ± 0.04*
14	0.7 ± 0.01*	0.4 ± 0.06	1.75 ± 0.2	0.7 ± 0.02*	1.8 ± 0.1*
21	0.8 ± 0.03*	0.3 ± 0.06*	2.67 ± 0.1	0.7 ± 0.01*	1.8 ± 0.1*

Statistical significance of differences compared to control (0 day): *significant at P< 0.05.
Values are means of three replicates ± standard deviation (SD)

Table 6: Amino acids, soluble protein and soluble carbohydrate contents of banana plantlets under the influence of several acclimatization periods with or without chitosan treatment.

Treatments (days)	Amino acids (mg/g DW)	Soluble protein (mg/g DW)	Soluble carbohydrates (mg/g DW)
Without chitosan			
0	12.42 ±1.41	121.27 ± 3.39	34.43 ±0.58
3	11.86 ±0.88	99.30 ±2.98*	30.66 ± 0.79
7	5 ± 1.45*	56.1 ± 6.03*	19.09 ±1.72*
10	2 ± 0.16*	62.65 ± 0.44*	31.5 ± 1.58*
14	3.26 ± 0.15*	68.74 ± 1.37*	34 ± 1.8
21	1.68 ± 0.1*	74.89 ±2.19*	39.72 ±1.36*
With 0.1% chitosan			
0	14.67 ± 0.24	148 ± 4.5	49 ± 0.2
3	10.46 ±1.45*	100.5 ± 0.66*	42.8 ± 1.5*
7	12.17 ± 1.1*	111 ± 2*	31.5 ± 1.58*
10	5.75 ± 0.13*	68 ± 2.24*	43.75 ± 1.24*
14	2.35 ± 0.25*	68 ± 7.65*	37 ± 2.15*
21	2.92 ± 0.19*	119.55 ± 4.37*	42.65 ± 2.28*

* Means that differences were significant at p < 0.05, compared to control (0 day).
Values are means of three replicates ± standard deviation (SD)

Table 7: POX and SOD activities of banana plantlets under the influence of several adaptation periods with or without chitosan.

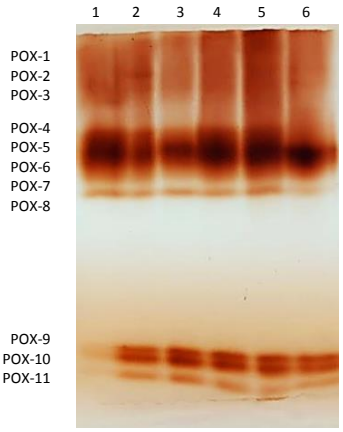
Adaptation time (days)	POX (mM g^{-1} FW)	SOD (mM g^{-1} FW)
Without 0.1% chitosan		
0	0.0153 ± 0.003	0.56 ± 0.1
3	0.018 ± 0.001	3.23 ± 1.5*
7	0.054 ± 0.005*	0.34 ± 0.07
10	0.06 ± 0.002*	0.9 ± 0.7
14	0.01±0.001*	0.6 ± 0.4
21	0.02 ± 0.004*	0.044 ± 0.001*
With 0.1% chitosan		
0	0.025 ± 0.001	0.7 ± 0.3
3	0.086 ± 0.0012*	1.4 ± 0.6*
7	0.06 ± 0.0004*	1.05 ± 0.008*
10	0.065 ± 0.0005*	1.4 ± 0.6*
14	0.05 ± 0.0004*	0.72 ± 0.4
21	0.023 ± 0.0003*	0.2 ± 0.1

* Means that differences were significant at p < 0.05, compared to control (0 day).

Values are means of three replicates ± standard deviation (SD).

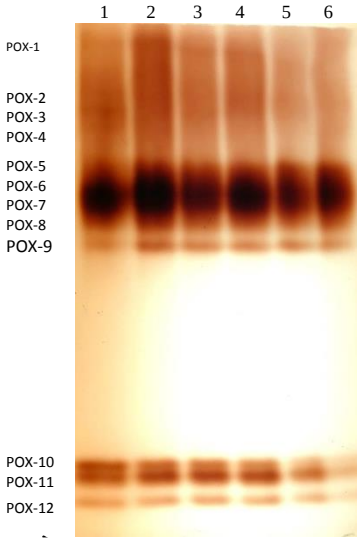
Under acclimatization periods, POX activity (Table 7) and POX staining intensities (Fig. 5) increased by the third day, and this continued until the fourteenth day. POX activity had decreased significantly again by the twenty-first day when compared to the *in vitro* obtained plantlets, regardless of the presence of chitosan or not. The increased activity of the POX can be attributed to an increase in the staining intensity of bands POX-1, POX-2 and POX-3 in the absence of chitosan (Fig. 5) and to an increase in the staining intensity of bands POX-4, POX-5 and POX-6 in the presence of chitosan (Fig. 6). In general, plants treated with chitosan resulted in an increase in the POX activity and a slight increase in the staining intensity of some bands such as POX-6.

The recorded trend of SOD was similar to what was observed for peroxidase, i.e. a significant increase in



Figgure 5: Native gel electrophoresis of POX isoenzye pattern of banana plantlets subjected for acclimatization for 0, 3, 7, 10, 14 and 21days without chitosan treatment, lanes 1, 2, 3, 4, 5 and 6, respectively.

SOD activity was from the third day of acclimatization, but it returned to decrease from the seventh day, and a significant decrease in values were recorded by twenty-first day (Table 7), when compared with values obtained at zero day of acclimatization. The recorded values of the superoxide dismutase for plantlets treated with chitosan were higher than those of the untreated plants (Tables 7).



Figgure 7: Native gel electrophoresis of POX isoenzye pattern of banana plantlets subjected for acclimatization for 0, 3, 7, 10, 14 and 21days with chitosan treatment, lanes 1, 2, 3, 4, 5 and 6, respectively.

Discussion

In this work, banana shoot tips containing meristematic domes surrounded by small leaf initials were used to establish shoot culture of banana on MS medium containing 5 mg/l BAP and 30 g/l sucrose as was reported by Sandoval & Müller 1987; Hassanein *et al*. (2011); Rodge *et al*. (2023). Initial banana explants were aseptically transferred to the same medium each three weeks (three times) to avoid tissue browning due to phenol accumulation as reported by Hassanein *et al*. (1999). Also, induction of *in vitro* shoot formation and multiplication was efficiently fulfilled on the same type of medium for three weeks, in agreement with Hassanein *et al.* (2005). Therefore, numerous shoots/shoot tip could be obtained for induction of root formation or commercial application. However, incubation of plant shoots under conditions of high relative humidity on artificial medium in glass jars may result in inhibition of some physiological functions and anatomical characteristics (Hazarika *et al*., 2006). Consequently, these plantlets would be desiccated and died unless essential precautions were taken to acclimatize them. Generally, acclimatization is carried out in greenhouse or field where irradiance is much higher and air humidity is much lower than those of *in vitro* culture jars. To avoid drastic changes between *in vitro* and *ex vitro* conditions, banana shoot formation and multiplication as well as acclimatization were carried out under the same physical conditions in tissue culture room. Thus, micropropagated banana plantlets could overcome the obstacles of *ex vitro* conditions in 21 days. Elisama *et al*. (2013) reported that the critical period for successful acclimatization was 20 days.

In bananas, establishment of extensive root formation in high frequency was essential prerequisite for successful acclimatization, and it depended on root formation procedure, IBA concentration, frequency of root formation and root number/shoot cutting (Hassanein *et al*. 2003, 2005, 2011; Kandha *et al*. 2022). Frequency of root formation was 100% when it was carried out *in vitro* on half strength MS medium containing 1 mg/l IBA, regardless the presence or absence of 0.1% (w/v) chitosan. On the contrary, Kandha et al. (2022)reported that combination between, BAP and auxins was necessary for successful shoot multiplication and root formation.

Root formation using *ex vitro* hydroponic procedure was carried out by dipping the explant in a solution containing IBA, it was used in a way similar to root formation on stem cuttings established by Zhao *et al*. (2022). Under these conditions, the highest root number was obtained using 1 mg/l IBA, it was recommended for successful acclimatization. The frequency of root formation using 1 mg/l IBA was lower than that of 2 mg/l IBA. Acclimatization can be improved by hormonal stimulation of root formation (Van Telgen *et al*., 1992). The frequency of plantlets survival using the *ex vitro* hydroponic culture (94%) was higher than that of *ex vitro* soil one (80%). This indicated that the availability of auxin to the bases of banana cuttings in the aqueous medium was better than that in the soil. In *Pyrus elaeagrifolia*, *ex vitro* root formation was significantly obtained by quick-dip of plant materials in 10 mM IBA (Aygun & Dumanoglu, 2015).

In this work, acclimatization was carried out under tissue culture room environment. Plantlets with *in vitro* formed roots showed increase in growth parameters during the acclimatization period, and these growth parameters were progressively increased when chitosan applied, this effect was extended even for 40 days under open conditions. Safana *et al*. (2022) reported that foliar application of chitosan during plantlets acclimatization increased plant survival. This

means that, abnormalities of *in vitro* grown banana plantlets might be repaired during acclimatization period (Shekhawat *et al*., 2021), especially when it was carried out under the influence of chitosan and high relative humidity (87%) under plastic bags. In other studies, efficient banana proliferation and acclimatization were established using 5 mg/l BAP in combination with 0.5 mg/l IBA and 25 mg/l chitosan (Veraplakorn & Kudan 2021; Kandha *et al*., 2022). According to our protocol, 100% of plantlets survival was obtained when root formation and acclimatization were combined in one stage under tissue culture room conditions where plants avoided changes in temperature and light intensity. On the other hand, 100% of plant death was detected when any of these acclimatization procedures was not applied.

In our study, during the first 10 days of *ex vitro* conditions, banana plantlets showed decrease in water content and slight increase in growth parameters. Elisama *et al*. (2013) found that *in vitro* formed leaves died within ten days of acclimatization, but active growth associated with the formation of new leaves were detected during the following days. Water loss was three times faster in *in vitro* grown plants compared to those grown in greenhouse (Brainerd *et al*., 1981). In our work, acclimatization of banana plantlets for more than ten days increased plantlets growth and raised of water contents, further increase in water content was recorded in the presence of chitosan. In *Malus pumila*, the highest water content was detected after transplantation for 3 weeks (Díaz-Pérez *et al*., 1995).

Generally, chlorophyll a and b contents of *in vitro* obtained plant materials may be higher or lower than that of *ex vitro* grown plants depending on light intensity and medium sugar content (Tichá *et al*., 1998). In this work, the plant was not exposed to different temperature or light intensities during acclimatization, since it was carried out under tissue culture room conditions. On the other side, transplanted banana plantlets were suffered from a decrease of water content due to inability to absorb their need of water because the *in vitro* formed roots did not practice water absorption before. Therefore, Chl.a, Chl.b, carotenoids and total pigments decreased within 10 days under *ex vitro* condition. Thereafter, these pigment fractions were increased as was detected in other plant species (Pospíšilová *et al*., 1998), that leading to perform efficient photosynthesis and plantlets growth (Elisama *et al*., 2013). Furthermore, net photosynthetic rate decreased during the first week after transplantation but increased thereafter, in agreement with results obtained by Van Huylenbroeck & De Riek, 1995; Pospíšilová *et al*., 1999. In our study, chitosan application increased all the growth parameters.

The high ability of banana plantlets to survive if the acclimatization process was carried out under tissue culture room conditions indicated that plantlets gained the ability to practice photosynthesis to the extent that allows the plants to grow within two weeks. In addition, plantlets formed new leaves and repaired the photosynthetic organs to carry out photosynthesis efficiently in agreement with the study of Pospíšilová *et al*. (1999). Consequently, plant contents of soluble carbohydrates and soluble proteins showed immediate and significant decrease upon transferring the plantlets to the *ex vitro* environment for 7 days and they increased by extending the acclimatization period. Values of amino acids, soluble carbohydrates and soluble proteins were higher in plantlets treated with chitosan than untreated plantlets. Application of chitosan increased organic fractions where they act as osmoregulators and/or up-regulator of many genes involved in carbohydrate transport and metabolism (Li *et al*., 2019; Abdel-Aziz *et al*., 2018). In other study, higher concentrations of chitosan inhibited the multiplication and growth of olive shoots grown *in vitro* (Darwesh *et al*., 2023).

Water deficiency during the first 10 days of banana transplantation resulted in over production of reactive oxygen species leading to reduce of plant growth, in agreement with Yang *et al*., 2009; Bistgani *et al*., 2017. In banana and other plant species, peroxidase and superoxide dismutases catalyze the dismutation of O_2^- to H_2O_2 and O_2 as well as H_2O_2 removal (Yang *et al*. 2009; Bistgani *et al*. 2017). Consequently, in our study, during transferring of banana plantlets from *in vitro* to *ex vitro* conditions, the activity of POX and SOD increased to control the increase in ROS and continued until suitable water content of the plantlets attained at the tenth day. Then, their activities decreased again. This work indicated that, increase POX activity was due to increase the staining intensity (expression) of some isoenzyme forms without the appearance of new one. Activity of POX and SOD of chitosan treated plantlets was higher than those of untreated ones. During water shortage, application of chitosan in banana and other plant species stimulated plant growth through increase plant water content, thereby it contribute to ROS scavenging activities (Guan *et al*., 2009) through increase the activities of antioxidant enzymes such as superoxide dismutase and peroxidase. When *Plantago almogravensis* plantlets were transferred to the *ex vitro* conditions, decrease of H_2O_2 content was detected and associated with increase of CAT and SOD activitie.

Conclusion

Generally, banana shoot tips were cultured *in vitro* for shoot multiplication and root formation. Plantlets were acclimatized under greenhouse or field conditions where irradiance is much higher and air humidity is much lower than the *in vitro* conditions. This work described alternative procedures for decreasing plantlets mortality and shorten the acclimatization periods. The determined growth and physiological parameters indicated that the efficiency of the acclimatization process increased if chitosan was used due to increase plant water content and ROS scavenging through increase the activities of antioxidant enzymes such as superoxide dismutase and peroxidase. The results also indicated that root formation can be combined with acclimatization in one stage if they were carried out under conditions of high relative humidity (87%) even for 10 days, 25 ± 2 °C and 16 h photoperiod with 100 µmol $m^{-2}s^{-1}$.

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