EFFECT OF MICROPROPAGATION CONDITIONS ON ADVENTITIOUS BUDS FORMATION AND THE CIRCADIAN EXPRESSION OF THE ACO013229.1 GENE IN *ANANAS COMOSUS*

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Smooth cayenne pineapple cultivar is considered the most suitable variety for the climatic conditions in Egypt, in addition to its distinctive flavor and ability for canning. To meet market demand, large quantities of plant materials are required, which cannot be obtained *via* traditional breeding methods. As a result, an *in vitro* technique was designed to increase the multiplication rate, rooting, and acclimatization of this unique pineapple variety. Thidiazuron (TDZ) at 2.0 mg/l proved to be superior for direct organogenesis rate. Half strength Murashige and Skoog (MS) medium containing 1.0 mg/l indole-3-butyric acid (IBA) in combination with 0.5 mg/l naphthalene acetic acid (NAA) improved the number and length of roots. Organogenesis has been accelerated from *in vitro* derived leaves and developed to healthy plantlets, which were acclimatized in the greenhouse. In order to investigate the effect of micropropagation on circadian rhythm, the circadian expression of Aco013229.1 was compared, which belongs to the MADS-box gene family, between the *in vitro* propagated plantlets and the *in vivo*-grown plants. The unaffected expression pattern of Aco013229.1 proposed that *in vitro* micropropagation did not affect the circadian cycling; hence, the CAM photosynthesis process was not interrupted. Moreover, the circadian expression of Aco013229.1 of the *in vitro* and *in vivo*-grown plants showed a similar pattern, strongly pointing at a stable circadian rhythm of the micropropagated plants and thus a well-maintained CAM photosynthesis. This gene family plays a significant role in a number of biological processes especially flowering.

Keywords: *Ananas comosus*, smooth cayenne, circadian rhythm, *in vitro*, organogenesis, thidiazuron

INTRODUCTION

The pineapple *Ananas comosus* Merr. is the world's most important tropical fruit (Chen et al., 2019). It is the only Bromeliaceae genus that has been successfully cultivated (Huihuang et al., 2020). Its ability to use water efficiently by utilizing the crassulacean acid metabolism (CAM) photosynthesis process makes it a good fit for dry lands (Ming et al., 2015 and Zhang et al., 2020). In CAM photosynthesis, plants store carbon dioxide during night and use it in photosynthesis during day to avoid losing water *via* gaseous exchange in opening stomata, thus, increasing the plant`s ability to tolerate drought for longer periods of time. However, the molecular regulators of CAM photosynthesis are still largely unexplored. The MADS-box gene family, for example is known for its capacity to attach to DNA (Ming et al., 2015). This process is orchestrated by the circadian clock (Ming et al., 2015 and Zhang et al., 2020). Flower formation in pineapple plants is influenced by two key factors: short days and low temperatures (Maruthasalam et al., 2010). The chemical composition of pineapple (sugars, organic acids, minerals, fiber, aromatic compounds, vitamins, amino acids, flavonoids, carotenoids, etc.) depends greatly on the variety. It contains very good amounts of vitamin B6 (pyridoxine), niacin, riboflavin and folic acid. Moreover, pineapple fruit is rich in minerals with high biological activity (Assumi and Jha, 2021). In addition, as compared to other cultivars, smooth cayenne had the largest content of bioactive compounds, antioxidant capacities, and bromelain production in terms of biochemical properties (Viana et al., 2013).

Traditional vegetative propagation of pineapple causes disease spread, lack of uniformity, and inadequacy for commercial processing, both of which create a bottleneck in meeting global pineapple fruit demand. Obtaining materials from the pineapple sucker, crown, and slips using conventional techniques will take up to 16 to 18 months after the fruit is harvested. Furthermore, plant material transported from other countries is very pricey (Hamid et al., 2013). Besides which, by using the traditional propagation process, the multiplication rate of pineapple is low. Suppliers are struggling to satisfy the high demand for pineapple planting supplies as a result of this. *In vitro* propagation, on the other hand, has emerged as a vital solution for obtaining disease-free, rapid, standardized, and mass production of pins. Many authors have reported successful production of pineapple via micropropagatiom (Firoozabady and Gutterson, 2003 and Demissie et al., 2009). Hence, the multiplication rates and tissue culture techniques need to be improved for pineapple (Almeida et al., 2002).

As a result, the current research looked into the effect of plant growth regulators on *in vitro* proliferation, rooting, and greenhouse acclimatization of pineapple plantlets, as well as the expression of the flowering gene.

MATERIALS AND METHODS

Explants of pineapple (*Ananas comosus* cv. smooth cayenne) were obtained from a pineapple plantation on the Cairo-Alexandria desert road's horticulture field. The first step after removing buds from the parent plant is disinfestation, which removes any microorganisms present and reduces the risk of fungal and bacterial contamination.

These buds were sterilized in a laminar flow chamber under completely aseptic conditions, eliminating excess tissue before being placed into the culture medium. The buds were carefully submerged in fungicide for 10 min after being cleaned with sterilized water to remove dust and dry matter. Pineapple buds were sterilized for 20 min with 30% Clorox (5.2% sodium hypochlorite solution) and then 0.2% mercuric chloride ($HgCl_2$) for 10 min with a few drops of Tween 20, before being rinsed three times with sterile distilled water.

Murashige and Skoog (1962) basal salts were used to make the media, which included 2.7 g/l (w/v) phytagel and 30 g/l sucrose. Prior to phytagel supplementation and homogenization, the pH of the medium was adjusted to 5.8 with 1 N NaOH or 0.1 N HCl. After dispensing 40 ml into jars, they were autoclaved for 20 min at 1.06 kg/cm^2 and 121°C. Sterilized buds were cultured on MS medium without plant growth regulators and incubated at 25±2˚C under 16 hours photoperiod provided by white fluorescent lamps.

1. Effect of Three Various Cytokinins on the Mean Number of Adventitious Buds from Leaf (Organogenesis Process)

For this experiment (organogenesis process), regenerated leaves from bud cultures were used as explants. The organogenesis medium was MS basal salts with vitamins supplemented with 30% sucrose, 2.7 g/l phytagel, thidiazuron (TDZ; N-phenyl-N′-1,2,3-thidiazol-5-yl urea), Kinetin (KIN; 6-furfurylaminopurine) and 6-(4-Hydroxy-3-methylbut-2-enylamino) purine (Zeatin) at 0.0, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l. After six and twelve weeks, mean number and length of adventitious buds/explant were recorded.

2. Influence of Light Intensity on the *in Vitro* Growth and Development (Proliferation Stage)

The aim of this experiment was to examine the influence of light intensity on the growth of pineapple at the multiplication stage. Cluster (contains three shoots) were grown on MS basal nutrient medium supplemented with 2 mg/l 6-benzyl adenine (BA), 30 g/l sucrose, and 2.7 g/l phytagel. At 16 hours photoperiod a day, all culture jars were incubated at 25 ± 2ºC in light provided by white fluorescent tubes with intensities of around 1000, 2000, 3000, or 4000 lux. The intensity of light emitted was measured by digital lux meter. Mean number of axillary shoots/ explant, mean length of axillary shoots and mean length of leaves were recorded.

3. Effect of MS Strength with Auxins on the Rooting Stage

The aim of the rooting stage is to prepare the plantlets for the establishment outside the artificial closed atmosphere of culture vessels. Each individual shoot was separated and transferred to a rooting medium in a culture tube or jar for root induction. Full-strength or half-strength MS basal medium with vitamins, supplemented with or without growth regulators, was used as the rooting medium. Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) at 0.25, 0.5, 1.0, 1.5, and 2.0 mg/l were used sparingly in the medium. After six weeks, the mean number of roots per shoot, root length, and shoot height were recorded.

4. Effect of MS Strength with IBA and NAA and Their Combinations on Enhancing Rooting

Each medium supplemented with IBA at 0.0, 0.5, and 1.0 mg/l in combination with NAA at 0.0, 0.25, and 0.5 mg/l was used to reinforce the rooting of pineapple using MS medium at two salt concentrations (full and half). After six weeks, the mean number of roots/shoot, root length, and shoot height were recorded.

5. Effect of Acclimatization Mixture on the Acclimatization Stage

The adaptation stage includes shifting the plantlets from the aseptic culture system to the greenhouse's free-living environment, and then to the final site. Rooted plants were planted in pots containing a sterile soil of peat moss and sand in proportions of 1:1, 1:2, 2:1, and 2:2, respectively, then covered with a transparent polypropylene package and kept in the greenhouse for six weeks. After two weeks, one pore in the package was created, followed by another at four weeks, and eventually, at the end of six weeks, the package was removed and the plants were transferred to the open field. After six weeks, the percentage of survived plantlets, the mean length of plantlets, and the number of leaves per plantlet were noted.

6. RNA Extraction

To isolate total RNA from pineapple plantlets, samples (approximately 20 mg) were frozen in liquid nitrogen. Using mortar and piston, samples were homogenized and transferred to a 1.5 ml centrifuge tubes. Further processing was performed using the RNeasy R Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions. The RNA was eventually eluted with RNase-free water.

7. cDNA Synthesis

cDNA was synthesized using SuperScriptTMII Reverse Transcriptase (RT) (Invitrogen) following the supplier’s instructions. In short, 1 µL Oligo (dT) primers, 500-1000 ng RNA and 1 µL dNTP mix was incubated for 5 min at 65°C. Reaction buffer (1×) and 10 µM DTT were added. Samples were

incubated at 42°C before addition of the RT. The synthesis was performed over 60 min with heat inactivation of the enzyme for 15 min at 70°C.

8. Quantitative Real-Time PCR (qRT-PCR)

Transcript analysis was performed by using cDNA corresponding to 500 ng RNA from three biological replicates. SYBR green assays were developed using iQ™ SYBRR® Green Supermix (Bio-Rad, Hercules, USA) with gene-specific primers. The reaction set up was adjusted to a total volume of 25 µl with 12.5 µl iQ SYBR Green Supermix, 1 µl of each primer at 10 µM and 10 µl cDNA template. PCR was performed on 'iQ5 multicolor real-time PCR detection system (Bio-Rad). Expressions were calculated using the CT method (Schmittgen and Livak, 2008). The gene of an expressed protein (Ananas β-Actin) served as internal control. This was previously proposed as reference gene (Luan et al., 2020).

9. Data Analysis

Analysis of variance (ANOVA), a statistical analysis program, was used to perform data variance analysis. Duncan's multiple range test (Duncan, 1955). was used to see if the differences between means for all treatments were significant at the 5% level. At $P \leq 0.05$, means preceded by the same letter are not substantially different.

RESULTS

However, there are several concerns with this plant's proliferation. Pineapple is limited by poor efficiency, disease vulnerability, and higher development costs. Micropropagation methods have been successfully used to resolve certain limitations in different crops, as well as ornamental and horticultural plants, in recent years. After eliminating the dust from the buds and disinfecting them with different disinfectant materials such as $HgCl_2$ and Clorox, the buds were found to be healthy (70% survival) and contamination-free (100%), and they were then cultured in MS medium.

1. Effect of Three Various Cytokinins on the Number of Adventitious Buds from Leaf (Organogenesis Process)

Data in table (1) and fig. (1) illustrate the effect of MS medium containing different concentrations of cytokinins on the differentiation of organs (adventitious buds) from *in vitro* derived young leaves. All concentrations of TDZ, KIN and Zeatin showed shoot proliferation from leaves ranged between 10 and 61 buds/ explant. For instance, after 6 weeks, MS medium augmented with 2.0 mg/l TDZ produced the highest number of shoots development from the leaves and formed a cluster of 44 adventitious buds/explant, followed by Zeatin at 2.0 mg/l, compared with the other treatments. The control treatment and the low concentrations of KIN recorded the lowest number of buds (10 buds/ explant). However, the length of the shoots in all treatments was the same (0.5 cm).

Table (1). Effect of various concentrations of TDZ, KIN, and Zeatin on the differentiation of pineapple leaves into shoots.

Cytokinin conc. (mg/l)		Mean number of adventitious buds/explant		Mean length of adventitious buds (cm)	
		6 weeks	12 weeks	6 weeks	12 weeks
Control		10^{l}	18^{l}	0.5	4.50^{i}
TDZ	**0.25**	15^{j}	25^{h}	0.5	7.70^{b}
	0.50	18^{i}	28^{g}	0.5	8.20^{a}
	1.00	27^{f}	35^{d}	0.5	4.00^{k}
	2.00	44^{a}	61^{a}	0.5	8.20^{a}
	4.00	40^{c}	31^{e}	0.5	6.60^{d}
KIN	**0.25**	10^{l}	11^{n}	0.5	3.30^{l}
	0.50	10^{l}	16^{m}	0.5	5.10^{g}
	1.00	13^{k}	20_{k}	0.5	4.10^{j}
	2.00	29^{e}	40^{c}	0.5	6.10^{e}
	4.00	19^{h}	25^{n}	0.5	4.13^{j}
Zeatin	**0.25**	13^{k}	20^{k}	0.5	6.00^{f}
	0.50	15^{j}	23^{i}	0.5	7.10^{c}
	1.00	25^{g}	30^{f}	0.5	3.30^{l}
	2.00	41^{b}	52^{b}	0.5	6.00^{f}
	4.00	35^{d}	21^{j}	0.5	5.00^{h}

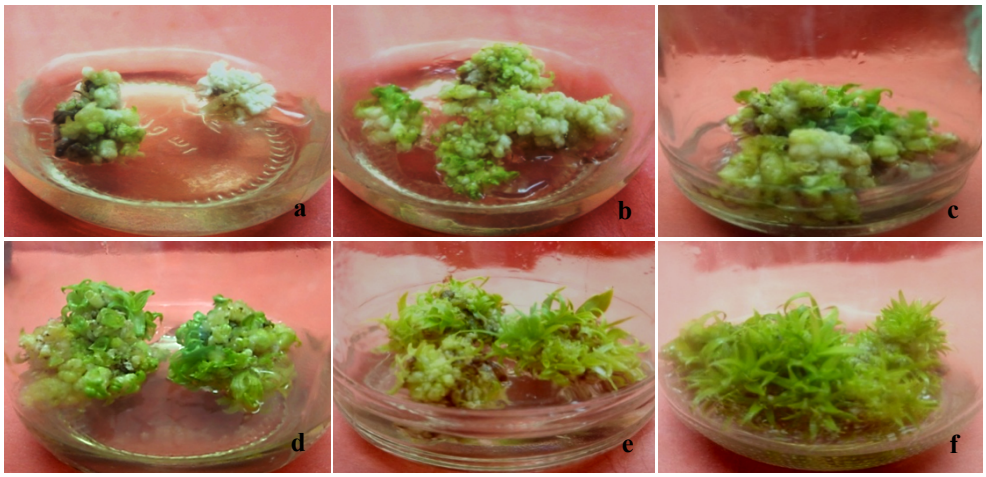

Fig. (1). *In vitro* regeneration *via* direct organogenesis from proximal leaf explant of pineapple smooth cayenne cultivar. **a.** Swilling of leaf buds. **b.** and **c.** Organogenesis induction from explants cultured on medium containing 2.0 mg/l TDZ. **d.** Development of organs cultured on medium containing 2.0 mg/l TDZ after 6 weeks. **e.** Developed shoots. **f.** Advanced shoot formation after the second subculture.

2. Influence of Light Intensity on *in Vitro* Growth and Development (Proliferation Stage)

The effect of light intensity (500, 1000, 2000, 3000, and 4000 lux) on the growth and production of shoots and leaves of pineapple cultured *in vitro* is represented in table (2) and fig. (2). When pineapple shoots were incubated under white fluorescence lamp at intensities of 4000 and 3000 lux (for 16 hours daily) at 25±2ºC, the maximum significant shoot number/explant was achieved with significant difference in between (61 and 48 shoots/explant, respectively).

Table (2). Effect of light intensity on growth and development of pineapple cultured *in vitro.*

Light intensity (lux)	Mean number of axillary shoots/explant	Mean length of axillary shoot (cm)	Mean length of leaves (cm)
500	11^{e}	5.3^{a}	4.06^{e}
1000	25^{d}	5.0^{b}	5.20^{d}
2000	40^{c}	4.4^{c}	6.10^{c}
3000	48^{b}	4.0^{d}	6.40^{b}
4000	61^{a}	3.5^{e}	7.06^{a}

Fig. (2). Proliferation rate of pineapple under 4000 lux *in vitro*.

3. Effect of MS Strength (Full MS and ½ MS) with IBA and NAA on Mean Number and Length of Roots and Mean Shoot Height during Rooting Stage

All of the shoots treated with IBA and NAA either full or half strength MS medium have a substantial impact on the mean number of roots/ explant mean length of roots (cm), mean and shoot height (cm), according to the data in (Table 3) and (Fig. 3). In comparison to complete MS medium, 1.5 mg/l NAA yielded the highest number of root/shoot (10.7) when used with 1/2 MS (4.5).

Table (3). Effect of full and half strength MS medium with different concentration of IBA and NAA on rooting of pineapple shoots.

Auxin conc. (mg/l)		Mean number of roots/shoot		Mean length of roots (cm)		Mean shoots length (cm)	
		Full MS	½ MS	Full MS	½ MS	Full MS	½ MS
Control		2.0^{i}	6.0^{j}	0.25^{f}	0.5^{f}	2.0^{f}	2.60^{g}
IBA	**0.25**	1.0^{j}	7.0^{h}	0.50^{d}	0.8^{c}	2.4^{c}	3.30^{c}
	0.50	2.2^{g}	6.5^{i}	0.75^{a}	0.6^{e}	1.9^{g}	2.70^{f}
	1.00	2.9^{d}	8.0^{e}	0.71^{b}	0.8^{c}	2.1^{e}	2.80^{e}
	1.50	2.3^{f}	7.23^{f}	0.41^{e}	0.7^{d}	2.1^{e}	2.60^{g}
	2.00	2.1^{h}	7.0^{h}	0.41^{e}	0.7^{d}	2.0^{f}	2.33^{h}
NAA	**0.25**	2.5^{e}	7.1^{g}	0.41^{e}	0.8^{c}	1.9^{g}	2.26^{h}
	0.50	4.4^{b}	8.9^{d}	0.53^{c}	1.3^{a}	2.7^{a}	4.20^{a}
	1.00	4.5^{a}	10.0^{b}	0.41^{e}	1.1^{b}	2.5^{b}	3.40^{b}
	1.50	4.5^{a}	10.7^{a}	0.41^{e}	0.8^{c}	2.2^{d}	3.26^{c}
	2.00	4.0^{c}	9.0^{c}	0.41^{e}	0.7^{d}	2.0^{f}	2.90^{d}

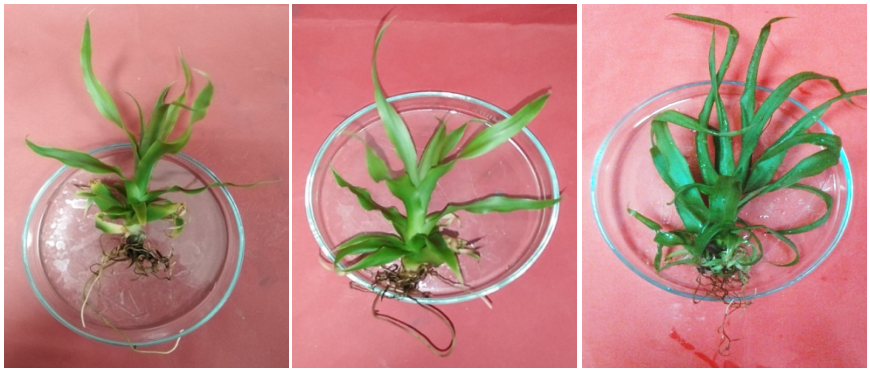

Fig. (3). Rooting of pineapple in half-strength MS basal medium with 1.5 mg/l IBA.

4. Effect of MS strength (Full MS and ½ MS) with IBA, NAA and their combinations on enhancing the rooting of shoots

The effect of full and ½ strength MS medium fortified with IBA in combination with NAA on enhancing root formation of pineapple was reported in table (4) and fig. (4). Data revealed that 1.0 mg/l IBA with 0.5 mg/l NAA was the optimum treatment. This auxin combination in ½ strength MS medium improved the number of roots (14.0 roots/shoot), root length (8.0 cm), and shoot height (12.1 cm), when compared to full strength MS medium, which produced 8.0 roots/shoot with length of 4.9 cm and 6.1 cm shoot height.

Table (4). Effect of MS medium (full and half strength) containing IBA in combination with NAA on enhancing pineapple roots.

Auxin conc. (mg/l)		Mean number of roots/shoot		Mean length of roots (cm)		Mean shoot height (cm)	
IBA	**NAA**	**Full MS**	**½ MS**	**Full MS**	**½ MS**	**Full MS**	**½ MS**
0.00	**0.00**	2.0^{i}	2.3^{h}	0.25^{i}	0.5^{i}	2.3^{e}	2.2^{i}
	0.25	2.9^{g}	2.9^{g}	0.4^{h}	0.7^{h}	1.9^{g}	3.2^{f}
	0.50	4.4^{c}	6.0^{d}	0.5^{g}	1.0^{g}	2.7^{c}	3.8^{d}
0.50	**0.00**	2.2^{h}	3.5^{f}	0.75^{e}	1.2^{f}	1.9^{g}	2.3^{h}
	0.25	4.0^{d}	7.0^{c}	1.0^{c}	1.8^{c}	2.5^{d}	3.7^{e}
	0.50	3.0^{e}	5.0^{e}	0.9^{d}	1.5^{d}	4.3^{b}	5.1^{b}
1.00	**0.00**	2.9^{f}	6.0^{d}	0.7^{f}	1.7^{e}	2.1^{f}	3.1^{g}
	0.25	5.0^{b}	9.0^{b}	2.0^{b}	3.8^{b}	4.3^{b}	4.9^{c}
	0.50	8.0^{a}	14^{a}	4.9^{a}	8.0^{a}	6.1^{a}	12.1^{a}

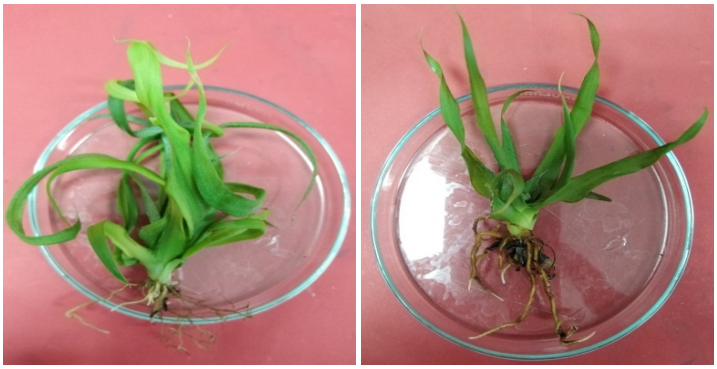

Fig. (4). Rooting stage of pineapple plantlets growing on MS medium with 1.0 mg/l IBA plus 0.5 mg/l NAA.

5. Effect of Soil Mixture on the Plantlets Acclimatization Stage

Except for those adapted on peat moss, transfer of pineapple plantlets with sterile roots to greenhouse conditions demonstrated nearly 100% survival success for all treatments. Table (5) and fig. (5) show the outcome of peat moss: sand 2:1 and 2:2, which scored 85.3 and 76.6%, respectively. Plants that adapted to 1 peat moss: 1 sand or 1 peat moss: 2 produced the most leaves (10 and 9 leaves/plantlet) and the tallest adapted plantlets of about 5 cm were ordered and cultured in the same mixture.

Table (5). Effect of media composition (peat moss and sand) on acclimatization of pineapple.

Soil mixture	Survival percentage (%)	Mean plant height (cm)	Mean number of leaves/plant
Peat moss: sand (1:1)	100.0[a]	5.00[a]	10[a]
Peat moss: sand (1:2)	100.0[a]	5.00[a]	9[b]
Peat moss: sand (2:1)	85.3[b]	2.66[b]	8[c]
Peat moss: sand (2:2)	76.6[c]	2.30[b]	6[d]

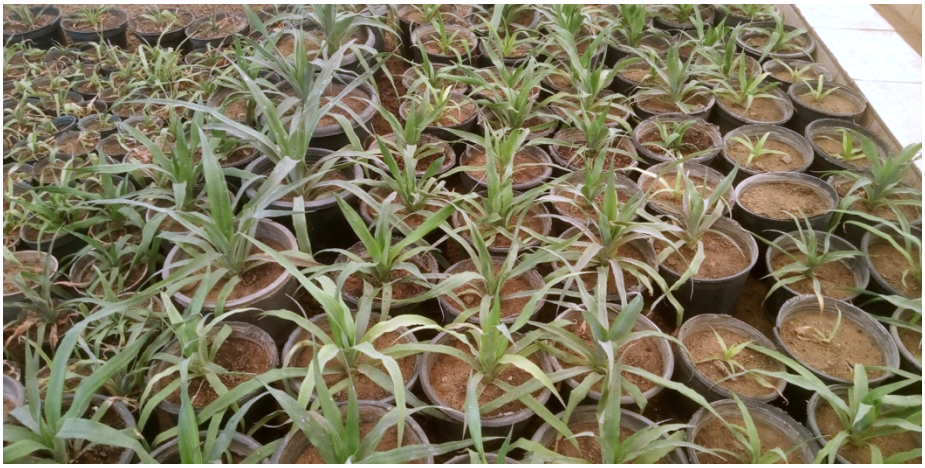

Fig. (5). Acclimatization of *in vitro* pineapple plantlets transplanted into peat moss: sand (1:1) after 12 weeks.

6. Micropropagation does not have an effect on the circadian rhythm.

In order to investigate the effect of micropropagation on circadian rhythm, the circadian expression of Aco013229.1 was monitored, which belongs to the MADS-box gene family. This family plays a significant role in a number of biological processes especially flowering. The members of this family share two highly conserved domains that encode for DNA-binding function. The gene expression of Aco013229.1 showed a circadian rhythm in pineapple plants that peaked around 10 am and significantly declined at 4 pm. Therefore, it was used to test the circadian cycling of the *in vitro* propagated plantlets. The qRT-PCR was used to measure the expression of Aco013229.1 in the *in vitro*- and *in vivo*-grown plants at two time points; 10 am and 4 pm over two days. The expression pattern of Aco013229.1 in both *in vitro*- and *in vivo*-grown plants peaked at 10 am and declined at 4 pm similarly (Fig. 6). This proposes that *in vitro* micropropagation did not affect the circadian cycling, hence, the CAM photosynthesis process is not interrupted.

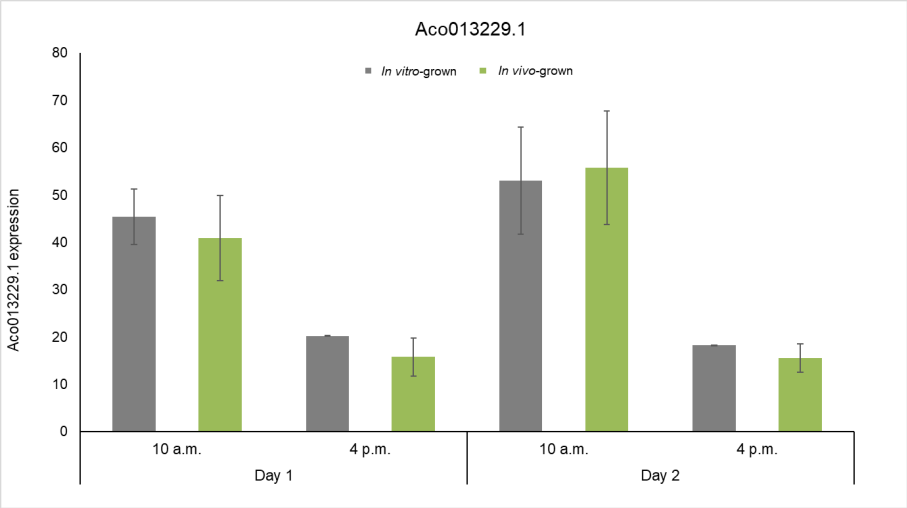

Fig. (6). Expression pattern of Aco013229.1 in *in vitro*- and *in vivo*-grown pineapple plantlets showing circadian cycling.

DISCUSSION

The *in vitro* technique's performance as a tool for plant propagation is highly dependent on the properties of the media utilized (Saad and Elshahed, 2012 and Arab et al., 2014). Explants grown *in vitro* have comparable basic needs as whole plants. As a result, George and De Klerk (2008) proposed that the culture media supply not only macro and micro nutrients, but also carbohydrate in the form of sucrose to replace carbon that would otherwise be acquired from the atmosphere. When vitamins, amino acids, and plant growth regulators are included in the culture media, better results will be attained (Kadhimi et al., 214 and Swamy et al., 2014). The medium used in this study has all of these components.

The presence of TDZ was found to be critical for organogenesis of pineapple, and this finding is consistent with Hassan et al. (2017), who found that MS medium supplemented with 1.0 mg/l TDZ combined with auxin produced the highest proportion of direct shoot buds and direct embryos formation. TDZ is a phenylurea-type compound with cytokinin-like physiological activity (Sakakibara, 2004). Leaf bases, according to Firoozabady and Moy (2004), may include meristematic regions or newly formed tissue with rapidly dividing cells that are amenable to morphogenesis in tissue culture. TDZ treatments also improved endogenous auxin, ethylene, and ABA levels (Murthy et al., 1995 and Hutchinson et al., 1996). Interestingly, combining 0.5 mg/l TDZ with 1.0 mg/l BA improved peroxidase activity during budding of date palm cv. Hillawi, where peroxidase activity was linked to the formation of more buds (Al-Mayahi, 2014). These findings were consistent with those of Taha et al. (2021), who used various

combinations of cytokinins such as N6-(2-isopentenyl) adenine (2iP), KIN, BA, and others on three date palm inflorescences (TDZ). In all three cultivars, TDZ alone or in combination with BA was found to be superior for direct organogenesis, so a new TDZ-BA combination was tested. TDZ at 2.0 mg/l could induce shoot in *Urginea altissima* leaf tissues (Baskaran et al., 2018), 1.5 mg/l in *Passiflora miniata* (Carvalho et al., 2019), and 1.0 mg/l in *Aloe vera* leaf tissues (Lavakumaran and Seran, 2014). TDZ at 0.5 and 5.0 µM was found to be optimum for inducing an average of 4–5 shoots per cotyledonary node in 93% of the cultures and 55 somatic embryos in 68% of the cultures by Chhabra et al. (2008**)**. According to Nsibande and Zhu (2017), the medium supplemented with 1.1 mg/l TDZ produced the highest shoot regeneration rate (75%) of *Hypoxis* species. Many scientists have attempted to reason out how TDZ works in plants. Dey et al. (2012), for example, believe that TDZ causes cells in the apical meristem to divide and multiply, then mature, resulting in bud differentiation. According to Mundhara and Rashid (2002), calcium stress triggers TDZ's ability to induce shoot bud production in the dark, which affects ethylene production. The metabolism of endogenous growth regulators is closely linked to the role of TDZ in morphogenesis.

The positive effect of high light intensity on the proliferation of pineapple shoots is confirmed by Chen et al. (2019), who found that using 1.0 mg/l BA + 0.1 mg/l NAA and a light intensity of 10 µmol m^{-2} s^{-1} resulted in the highest callus proliferation index (93.15%). Under a light intensity of 45 µmol m^{-2} s^{-1}, the best shoot proliferation rates were on media of either 1 mg/l BA + 0–0.4 mg/l NAA (65.57–81.01%). When adventitious shoots were cultured on MS medium with 0.4 mg/l NAA + 0.4 mg/l IBA, the maximum root length (15.57 mm) and the highest rooting frequency (17 roots per shoot) were obtained. Plant hormones influence changes in plant physiology and morphogenesis that are caused by light intensity or quality (Kissoudis et al., 2017).

The ratio of auxin to cytokinin during *in vitro* propagation might be important in inducing the morphogenic response in higher plants (García et al., 2008). Explants, in general, require cytokinin to develop and auxin to produce roots. Auxins and cytokinins are crucial for regulating growth and promoting callus development in micropropagation.

Auxins like NAA, IBA, or a combination of NAA and IBA to the medium helped improving pineapple rooting *in vitro*. The addition of IBA in combination with NAA to the culture media is one of the variables that contribute to the effectiveness of root formation and the production of healthy pineapple plantlets. The fact that these growth regulators can act in concert or synergistically for the induction of *in vitro* roots may have contributed to the significant increase in the mean number of roots produced when NAA and IBA were used together (Danso et al., 2008). The presence of NAA in the rooting medium increased the number of rooting embryos in date palms. The greatest root thickness was achieved using NAA or IBA at 0.4, 0.6, or 0.8 mg/l

with 60 g/l sucrose. NAA has an effect on the principal root length, and IBA has an effect on the lateral root length, according to Fatima and Anis (2012). Shoots of geranium were rooted on MS medium supplemented with 0.2 mg/l of NAA, according to Hutchinson et al. (1996). Similarly, Akin-Idowu et al. (2014) revealed that a half-strength MS basal medium supplemented with 0.9 mg/l NAA alone resulted in the maximum mean number of roots per shoot (approximately 7.9). In the same way, acclimating MD2 pineapple rooted plantlets in jiffy peatmoss pots resulted in maximum growth and greenhouse establishment (Danso et al., 2008). On the other hand, Amin et al. (2005) and Tavares et al. (2008) successfully constructed pineapple and bromeliad plantlets on sand. These findings were in line with those of Atawia et al. (2016), who found that adapted plants survived 100% of the time when peatmoss: sand 1:2 was used. The survival rate of pineapple plantlets with sterile roots in greenhouse conditions was nearly 100% (Zuraida et al., 2011).

Over two days, qRT-PCR was applied to evaluate the expression of Aco013229.1 in the *in vitro*- and *in vivo*-grown plants at two different time points: 10 am and 4 pm. Aco013229.1 expression peaked about 10 am and then dropped considerably by 4 pm. This finding fits as well with previous studies where the circadian expression of Aco013229.1 showed a similar pattern (Zhang et al., 2020). This gene family is involved in a variety of biological activities, particularly flowering. The aforementioned circadian expression of Aco013229.1 in the *in vitro*-grown indicates that the circadian clock is not interrupted as a result of micropropagation. In previous, studies the circadian clock orchestrated the CAM photosynthesis which, subsequently, increases the water use efficiency by pineapple plants (Ming et al., 2015 and Zhang et al., 2020). The findings of this study propose a functional circadian clock, which would maintain the CAM photosynthesis process, hence, helps the *in vitro*-produced plants to efficiently use water.

CONCLUSIONS

In this work, unique growth regulator sequences were developed that were incorporated in the nutritional medium for pineapple (*Ananas comosus*) direct organogenesis from *in vitro* leaves explant. TDZ is important cytokinin to add to pineapple induction and multiplication medium. Smooth cayenne variety has shown to be a promising cultivar for micropropagation and biotechnology, and its shoots have grown into vigorous plantlets that have acclimatized in the greenhouse.

Aco013229.1 gene expression in the *in vivo*- and *in vitro*-grown plantlets exhibited a similar pattern in this research. This suggests that *in vitro* micropropagation had no effect on circadian cycling, and therefore the CAM photosynthesis mechanism was unaffected. This photosynthesis strategy improves the ability of pineapple plants to efficiently use water. This family is involved in several biological processes, including flowering.

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تأثير ظروف الإكثار الدقيق على تشكيل البراعم العرضية وتعبير أحد جينات الساعة البيولوجية Aco013229.1 في نبات الأناناس

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يعتبر نبات الاناناس *Ananas comosus* صنف smooth cayenne من أكثر أصناف الأناناس ملائمة للظروف المناخية في مصر بالإضافة إلى نكهته المميزة وقابليته للتعليب. ولتلبية الطلب المتزايد في الأسواق فإن ذلك يستلزم توفير كميات من النباتات والتي يصعب إنتاجها وتوفيرها بإستخدام طرق التربية والإنتاج التقليدية. ونتيجة لذلك فقد تم تصميم بروتوكول للإكثار المعملي لهذا الصنف المميز من الأناناس لزيادة معدل التضاعف، التجذير والأقلمة في الصوب الزراعية. وقد ثبت أن أضافة ثيديازورون Thidiazuron (TDZ) بتركيز ٢,٠ ملليجرام لكل لتر أظهر أعلى تأثيرًا إيجابيًا في معدل التكشف المباشر للأعضاء النباتية. وأظهرت النتائج أيضًا أن استخدام بيئة النمو موراشيج وسكوج MS بنصف تركيز قوة الأملاح مضافًا إليها كلًا من أندول حمض البيوتريك indole-3-butyric acid بتركيز ١,٠ ملليجرام لكل لتر ونفثالين حمض الخليك naphthalene acetic acid بتركيز ٠,٥ ملليجرام لكل لتر قد أدت إلي الحصول على أعلى معدل زيادة في عدد وطول جذور النباتات. وقد تمت عملية تخليق الأعضاء معمليًا بدءًا من استخدام الأوراق وتطورت هذه الأعضاء حتى الوصول الى نبات كامل في الصوب الزراعية. ولاختبار تأثير الإكثار المعملي على الساعة البيولوجية لنباتات الأناناس فقد تم قياس التعبير الجيني للجين Aco013229.1 في النباتات المنزرعة في الحقل والنباتات المنتجة معمليًا. والمعروف عن هذا الجين أنه جين ينتمي للعائلة الجينية MADS-box والتي تؤثر في العديد من العمليات الحيوية في النبات مثل التزهير. وقد أظهرت النتائج أن الإكثار المعملي لنباتات الأناناس لم يؤثر سلبيًا على التعبير الجيني للجين Aco013229.1 وذلك بعد مقارنة نتائج النباتات التي تم إكثارها معمليًا بالنباتات المنزرعة في الحقل. وبناءً على هذه النتائج يمكن استخلاص أن الساعة البيولوجية لنباتات الأناناس لم تتأثر بالإكثار المعملي وبالتبعية فإن عملية البناء الضوئي CAM photosynthesis لم تتأثر أيضًا، وهي طريقة تستخدمها بعض النباتات ومنها الأناناس لتقليل فقد الماء وذلك عن طريق التحكم في مدة فتح وغلق الثغور. وفي ضوء ما سبق فإن الطريقة المتبعة في هذا البحث للإكثار المعملي لنباتات الأناناس تؤدي الى إنتاج نباتات بأعداد كبيرة مع المحافظة على قدراتها على التمثيل الضوئي التي تمكنها من إستخدام كميات أقل من الماء.