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

Manal E.A.E. Ahmed¹, Reda E.E. Abo El-Fadl¹, Mohamed N.S. Suliman² and Tamer M. Abd Elaziem*¹
¹Tissue Culture Unit, Department of Genetic Resources, Desert Research Center, Matareya, Cairo, Egypt
²Genetics and Cytology Unit, Department of Genetic Resources, Desert Research Center, Matareya, Cairo, Egypt
*E-mail: tamermahfouz86@yahoo.com

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 (Huikhun 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 micropropagation (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 disinfection, 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 (HgCl₂) 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/cm² 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-thiazol-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 ± 2°C 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™ SYBR® 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 HgCl₂ 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                |         |          |         |          |
| 0.25                   | 10<sup>l</sup> | 18<sup>j</sup> | 0.5 | 4.5<sup>l</sup> |
| 0.50                   | 18<sup>j</sup> | 28<sup>g</sup> | 0.5 | 8.20<sup>a</sup> |
| 1.00                   | 27<sup>f</sup> | 35<sup>d</sup> | 0.5 | 4.00<sup>k</sup> |
| TDZ                    | 2.00     | 44<sup>i</sup> | 61<sup>a</sup> | 0.5 | 8.20<sup>a</sup> |
|                        | 4.00     | 40<sup>g</sup> | 31<sup>c</sup> | 0.5 | 6.60<sup>d</sup> |
| KIN                    | 0.25     | 10<sup>l</sup> | 11<sup>a</sup> | 0.5 | 3.30<sup>j</sup> |
|                        | 0.50     | 10<sup>l</sup> | 16<sup>m</sup> | 0.5 | 5.10<sup>g</sup> |
|                        | 1.00     | 13<sup>k</sup> | 20<sup>h</sup> | 0.5 | 4.10<sup>j</sup> |
|                        | 2.00     | 29<sup>e</sup> | 40<sup>c</sup> | 0.5 | 6.10<sup>c</sup> |
|                        | 4.00     | 19<sup>h</sup> | 25<sup>a</sup> | 0.5 | 4.13<sup>j</sup> |
| Zeatin                 | 0.25     | 13<sup>k</sup> | 20<sup>a</sup> | 0.5 | 6.00<sup>f</sup> |
|                        | 0.50     | 15<sup>j</sup> | 23<sup>i</sup> | 0.5 | 7.10<sup>c</sup> |
|                        | 1.00     | 25<sup>g</sup> | 30<sup>f</sup> | 0.5 | 3.30<sup>j</sup> |
|                        | 2.00     | 41<sup>b</sup> | 52<sup>b</sup> | 0.5 | 6.00<sup>f</sup> |
|                        | 4.00     | 35<sup>d</sup> | 21<sup>j</sup> | 0.5 | 5.00<sup>a</sup> |

Fig. (1). *In vitro* regeneration via direct organogenesis from proximal leaf explant of pineapple smooth cayenne cultivar. a. Swelling 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±2°C, 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<sup>c</sup>                         | 5.3<sup>a</sup>                   | 4.06<sup>a</sup>           |
| 1000                 | 25<sup>d</sup>                         | 5.0<sup>b</sup>                   | 5.20<sup>d</sup>           |
| 2000                 | 40<sup>c</sup>                         | 4.4<sup>c</sup>                   | 6.10<sup>c</sup>           |
| 3000                 | 48<sup>b</sup>                         | 4.0<sup>d</sup>                   | 6.40<sup>b</sup>           |
| 4000                 | 61<sup>a</sup>                         | 3.5<sup>a</sup>                   | 7.06<sup>a</sup>           |

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 | 0.50 f  | 0.8 c | 2.4 e  | 3.30 g |
| 0.25               | 1.0 i   | 7.0 h | 0.50 d  | 0.8 c | 2.4 e  | 3.30 g |
| 0.50               | 2.2 g   | 6.5 j | 0.75 a  | 0.6 c | 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 g |
| 1.50               | 2.3 c   | 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 f | 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 h | 10.0 b  | 0.41 f | 1.1 b  | 2.5 b  | 3.40 b |
|                    | 1.50    | 4.5 a | 10.7 a  | 0.41 f | 0.8 c | 2.2 d  | 3.26 c |
|                    | 2.00    | 4.0 c | 9.0 e   | 0.41 f | 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 | 2.3b    | 0.25i | 0.5i    | 2.3e  | 2.2i |
| 0.25               | 0.50 | 4.4c | 6.0d    | 0.4b  | 0.7h    | 1.9g  | 3.2f |
| 0.50               |     |     |         |      |         |      |         |      |
|                    | 0.50 | 2.2h | 3.5f    | 0.75c | 1.2f    | 1.9g  | 2.3b |
|                    | 0.25 | 4.0d | 7.0e    | 1.0e  | 1.8e    | 2.5d  | 3.7c |
|                    | 0.50 | 3.0e | 5.0e    | 0.9d  | 1.5d    | 4.3b  | 5.1b |
| 1.00               |     |     |         |      |         |      |         |      |
|                    | 0.00 | 2.9f | 6.0d    | 0.7f  | 1.7c    | 2.1f  | 3.1g |
|                    | 0.25 | 5.0b | 9.0b    | 2.0b  | 3.8b    | 4.3b  | 4.9c |
|                    | 0.50 | 8.0a | 14a     | 4.9a  | 8.0a    | 6.1a  | 12.1a |

**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<sup>a</sup>       | 5.00<sup>a</sup>       | 10<sup>a</sup>              |
| Peat moss: sand (1:2)         | 100.0<sup>a</sup>       | 5.00<sup>a</sup>       | 9<sup>b</sup>              |
| Peat moss: sand (2:1)         | 85.3<sup>b</sup>        | 2.66<sup>b</sup>       | 8<sup>c</sup>              |
| Peat moss: sand (2:2)         | 76.6<sup>c</sup>        | 2.30<sup>b</sup>       | 6<sup>d</sup>              |

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.
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 (Lavakumar 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.
REFERENCES

Akin-Idowu, P.E., S.O.S. Akinyemi and D.O. Ibitoye (2014). Influence of medium type and growth regulators on in vitro micropropagation of pineapple (*Ananas comosus* (L.), var. smooth cayenne). African Journal of Plant Science, 8 (9): 450-456.

Al-Mayahi, A.M.W. (2014). Thidiazuron-induced in vitro bud organogenesis of the date palm (*Phoenix dactylifera* L.) cv. hillawi. African Journal of Biotechnology, 13: 3581–3590.

Almeida, W., G. Santana, A. Rodriguez and M. Costa (2002). Optimization of a protocol for micropropagation of pineapples. Rev. Bras. Fruit, 2: 296-300.

Amin, M.N., M.M. Rahman, K.W. Rahman, R. Ahmed, M.S. Hossain and M.B. Ahmed (2005). Large scale regeneration in vitro from derived callus cultures of pineapple (*Ananas comosus* L. (Merr.) cv. giant kew). International Journal of Botany, 1 (2): 128-132.

Arab, M.M., A. Yadollahi, A. Shojaeiyan, S. Shokri and S.M. Ghojah (2014). Effects of nutrient media, different cytokinin types and their concentrations on in vitro multiplication of G • N15 (hybrid of almond • peach) vegetative rootstock. Journal of Genetic Engineering and Biotechnology, 12: 81-87.

Assumi, S. P.T. and A.K. Jha (2021). Pineapple (*Ananas comosus* L. Merr.). book: In: “Tropical Fruit Crops: Theory to Practical”. (Ghosh, S.N. and R.R. Sharma Eds.), pp. 487-541.

Atawia, A.R., F.M. Abd El-Latif, S.F. El-Gioushy, S. Saied and O.M.A. Kotb (2016). Studies on micropropagation of pineapple (*Ananas comosus* L.). Middle East Journal of Agriculture, 5 (2): 224-232.

Baskaran, P., A. Kumari and J. Van Staden (2018). *In vitro* propagation via organogenesis and synthetic seeds of *Urginea alissima* (L.f.) Baker: a threatened medicinal plant. 3 Biotech, 8 (1). 18.

Carvalho, P.P., C.A. Antoniazzi, R.B. Faria, I.F. Carvalho, D.I. Rocha and M.L. Silva (2019). *In vitro* organogenesis from root explants of *Passiflora miniata* Mast., an Amazonian species with ornamental potential. Brazil Archives Biol. Technol., 62: e19170803.

Chen, Y.M, J.Z. Huang, T.W. Hou and I.C. Pan (2019). Effects of light intensity and plant growth regulators on callus proliferation and shoot regeneration in the ornamental succulent Haworthia. Botanical Studies, 60: 10.

Chhabra, G., D. Chaudhary, M. Varma, M. Sainger and P.K. Jaiwal (2008). TDZ-induced direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). Physiol. Mol. Biol. Plants, 14 (4): 347-353.

Danso, K.E., K.O. Ayeh, V. Oduro, S. Amiteye and H.M. Amoatey (2008). Effect of 6-benzylaminopurine and naphthalene acetic acid on *in vitro*
production of MD2 pineapple planting materials. Ghana. World Applied Sciences Journal, 3 (4): 614-619.

Demissie, Z.A., Tefera, W.M. Felleipe, A.T. Negawo and A.M. Adal (2009). In vitro multiplication of pineapple (Ananas comosus L.) and cardamom (Elletaria cardamomum) in Ethiopia. Proceeding of the Second Biennial Conference of Ethiopian Horticulural Science Society, Addis Ababa, Ethiopia, pp. 9-18.

Dey, M., S. Bakshi, G. Galiba, L. Sahoo and S. Panda (2012). Development of a genotype independent and transformation amenable regeneration system from shoot apex in rice (Oryza sativa spp. indica) using TDZ. 3 Biotech, 2: 233-240.

Duncan, D.B. (1955). Multiple range and multiple F tests. Biometrics, 11: 1-42.

Fatima, N. and M. Anis (2012). Role of growth regulators on in vitro regeneration and histological analysis in Indian ginseng (Withania somnifera L.) Dunal. Physiol. Mol. Biol. Plants, 18: 59-67.

Firoozabady, E. and N. Gutterson (2003). Cost effective in vitro propagation methods for pineapple. Plant Cell Report, 21: 844-850.

Firoozabady, E. and Y. Moy (2004). Regeneration of pineapple via somatic embryogenesis and organogenesis. In Vitro Cell Dev. Biol. Plant, 40: 67-74.

García, R.D., Z. Somonte, J. Zaldúa, A. Mena, R. López, R.M. Valdivia, A.D. Arencibia, K.Q. Bravo and P.D.S. Caligari (2008). Efficient regeneration and Agrobacterium tumefaciens mediated transformation of recalcitrant sweet potato (Ipomoea batatas L.) cultivars. Asia Pacific Journal of Molecular Biology and Biotechnology, 16 (2): 25-33.

George, E.F. and G.J. De Klerk (2008). The Components of Plant Tissue Culture Media I: Macro- and Micro-Nutrients. In “Plant Propagation by Tissue Culture”. 3rd Edition (George, E.F., M.A. Hall and G.J. De Klerk eds.). Springer, Dordrecht, The Netherlands, pp. 65–113.

Hamid, N.S., M.F.M. Bukhori and M. Jalil (2013). Direct and indirect plant regeneration of pineapple var. MD2 (Ananas comosus L.). Malaysian Applied Biology, 42 (1): 61-66.

Hassan, M.M., A.H.I. Abd-El Kareim, F.A. Hussein and I.M. Shams El-Din (2017). IBA and TDZ induced plant regeneration of date palm through immature female inflorescence culture. International Journal of Advances in Agricultural Science and Technology, 4: 1-16.

Huihuang, C., B. Hu, L. Zhao, D. Shi, Z. She, X. Huang, S. Priyadarshani, X. Niu and Y. Qin (2020). Differential expression analysis of reference genes in pineapple (Ananas comosus L.) during reproductive development and response to abiotic stress, hormonal stimuli. Trop. Plant Biol., 12: 67-77.

Hutchinson, M., S. Murch and P.K. Saxena (1996). Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in

Egyptian J. Desert Res., 71, No. 2, 191-208 (2021)
thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium × hortorum* Bailey). Journal of Plant Physiology, 149: 573-579.

Kadhimi, A.A., A.N. Alhasnawi, A. Mohamad, W.Y. Wan Mohtar and B.C.M.Z. Che Radziah (2014). Tissue culture and some of the factors affecting them and the micropropagation of strawberry. Life Science Journal, 11: 484-493.

Kissoudis, C., A. Seifi, Z. Yan, A.T.M. Islam, H. Van der Schoot, C.C.M. Van de Wiel, R.G.F. Visser, C.G. Van der Linden and Y. Bai (2017). Ethylene and abscisic acid signaling pathways differentially influence tomato resistance to combined powdery mildew and salt stress. Frontiers in Plant Science, 9 (7): 2009.

Lavakumaran, L. and T.H. Seran (2014). Effect of 6-benzyl-aminopurine and thidiazuron on *in vitro* shoot organogenesis of *Aloe vera* (L.) Burm. f. Chilean Journal Agricultural Research, 74: 497-501.

Luan, A., C. Chen, T. Xie, J. He and Y. He (2020). Methylation analysis of CpG islands in pineapple SERK1 promoter. Genes, 11 (4): 425.

Ming, R., R. Van Buren, C.M. Wai, H. Tang and M.C. Schatz et al. (2015). The pineapple genome and the evolution of CAM photosynthesis. Nature Genetics, 47 (12): 1435-42.

Maruthasalam, S., L.Y. Shiu, M. Loganathan, W.C. Lien, Y.L. Liu, C.M. Sun, C.W. Yu, S.H. Hung, Y. Ko and C.H. Lin (2010). Forced flowering of pineapple (*Ananas comosus* cv. Tainon 17) in response to cold stress, ethephon and calcium carbide with or without activated charcoal. Plant Growth Regul., 60: 83–90.

Mundhara, R. and A. Rashid (2002). Stimulation of shoot-bud regeneration on hypocotyl of *Linum* seedlings, on a transient withdrawal of calcium: effect of calcium, cytokinin and thidiazuron. Plant Science, 162: 211-214.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology, 15: 473-497.

Murthy, B.N.S., S.J. Murch and P.K. Saxena (1995). Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledons. Physiologia Plantarum, 94: 268-276.

Nsibande, B.E. and L. Zhu (2017). Developing an *in vitro* propagation method for mass production of medicinal *Hypoxis* species using bioreactors. Asian Journal of Plant Science and Research, 7 (4): 1-8.

Saad, A.I.M. and A.M. Elshahed (2012). Plant Tissue Culture Media. In: “Recent Advances in Plant *in Vitro* Culture” (Leva, A. and L.M.R. Rinaldi eds.), In Tech, Rijeka, Croatia, pp. 29-40.

Sakakibara, H. (2004). Cytokinin Biosynthesis and Metabolism. In: “Plant Hormones: Biosynthesis, Signal, Action”. 3rd Edition (Davies, P.J. ed.). Kluwer Academic Publishers, London, pp. 95-114.

Schmittgen, T.D. and K.J. Livak (2008). Analyzing real-time PCR data by
the comparative CT method. Nature Protocols, 3 (6): 1101–1108.
Swamy, M.K., S.K. Mohanty and M. Anuradha (2014). The effect of plant
growth regulators and natural supplements on in vitro propagation of
_Pogostemon cablin_ Benth. Journal of Crop Science and Biotechnology,
17: 1-7.
Taha, R.A., M.A. Allam, S.A.M. Hassan, B.M.M. Bakr and M.M. Bakr
(2021). Thidiazuron-induced direct organogenesis from immature
inflorescence of three date palm cultivars. Journal of Genetic
Engineering and Biotechnology, 19: 14.
Tavares, A.R., P. Giampaolo, S. Kanashiro, F.F.A. Aguiar and E.P. Chu
(2008). Effect of foliar fertilization KNO₃ in the acclimatization of
bromeliads grown in vitro. Horticultural Brasileira, 26 (2): 175-179.
Zhang, X., M. Fatima, P. Zhou, Q. Ma and R. Ming (2020). Analysis of
MADS-box genes revealed modified flowering gene network and diurnal
expression in pineapple. BMC Genomics, 21 (1): 8.
Zuraida, A.R., A.H. Nurul Shahnadz, A. Harteeni, S. Roowi, C.M.Z. Che
Radziah and S. Sreeramanan (2011). A novel approach for rapid
micropropagation of maspine pineapple (_Ananas comosus_ L.) shoots
using liquid shake culture system. African Journal of Biotechnology, 10
(19): 3859-3866.
تأثير ظروف الإكثر الدقيق على تشكيل البراعم العرضية وتعبير أحد جينات الساعة البيولوجية Aco013229.1

منال الصلاة على النبي أحمد، رضما السيد السيد أبو الفضل، محمد نادي سيد سليمان

وتمام محفوظ عبد العظيم

وجهة زراعة الأنسجة، قسم الأصول الوراثية، مركز بحوث الصحراء، المطرية، القاهرة، مصر

وجهة الوراثة والبيولوجيا، قسم الأصول الوراثية، مركز بحوث الصحراء، المطرية، القاهرة، مصر

يعتبر نبات الأناناس صنف Ananas comosus من أكثر أصناف smooth cayenne الأناناس ملاءمة للظروف المناخية في مصر بالإضافة إلى كثرة المميزه وقابلته للتعليب. واللبيئة الطلب المتزامن في الأماكن فان ذلك يستلزم توفير كميات من الدائيات والتي يصعب إنتاجها وتوفيرها باستخدام طرق التربية والانتاج التقليدية. ونتيجة لذلك فقد تم تجميع بروتوكل الابتكر المعملي لهذا الصف المميز من الأناناس لزيادة معدل التضاعف، التجنيب والأفرامل. وقد ثبت أن أضافة Thidiazuron (TDZ) متركبة 2.0 ملليجرام لكل تر تأثير أعلى إيجابياً في معدل التكذف المبكر للأعضاء النباتية. وأظهرت النتائج أيضاً أن استخدام البيويك naphthalene به مستخرج from MS موراشج وسوكنج Micheal 2.0 ملليجرام لكل تر تؤدي إلى الحصول على أعلى معدل زياادة في عدد الأوراق وتطور النباتات. وقد تم تم عملية تخليق الأعضاء عملياً بدلاً من استخدام الأوراق وتطور هذه الأعضاء حتى الوصول إلى نبات كامل في الصوب الزراعية. ولتأكيد تأثير الابتكر المعملي على الساعة البيولوجية لنباتات الأناناس فقد تم قياس التعبير الجنيني للجين 1 في Aco013229.1 على النباتات المنزوعة في الحقل والنباتات المنتجة معاملياً. والمعرفة عن هذا الجين الذي ينتمي لعائلة النباتات الموضوعة في النباتات مثل الزهور. وقد أظهرت النتائج أن النباتات المعالمة لم تؤثر سلباً على التعبير الجنيني للجين 1 Aco013229.1 وذلك بعد مقارنة نتائج النباتات التي تم إكثرها مع عالياً بالنباتات المنزوعة في الحقل. وبناءً على هذه النتائج يمكن استخلاص أن الساعة البيولوجية لنباتات الأناناس لم تتأثر بالإكثر المعطي، وبالتالي فإن المحاولة اليدوية هي طريقة تستخدمها بعض النباتات ومنها الأناناس لتكاثر قبل النكهة وذلك عن طريق التحكم في مدى فتح غلق الفروع. وفي وضع ما صبي فان الطرق المبسطة في هذا البحث للإكثر المعطي لنباتات الأناناس تؤدي إلى نتائج بجود كبيرة مع المحافظة على قدرتها على التمثل الديفوني التي تمكناها من استخدام كميات أقل من النكهة.

Egyptian J. Desert Res., 71, No. 2, 191-208 (2021)