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Research Article

Effect of dark incubation in germination of indirect date palm somatic embryos and conversion into plantlets

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Abstract All studies on date palm somatic embryogenesis have focused on germination in the presence of light while neglecting germination in darkness, which mimics the germination process of zygotic embryos within seeds. To improve the date palm micropropagation protocol, we investigated the effects of light and darkness incubation on the germination of indirect date palm somatic embryos and their subsequent conversion into plantlets. Darkness incubation emerged as a pivotal factor in the germination of indirect date palm somatic embryos and their successful conversion into plantlets. Darkness incubation significantly decreased the time required for the conversion of indirect somatic embryos into plantlets, halving the duration from 24 weeks to only 12 weeks. The micropropagation protocol was modified, consolidating the previous two distinct stages of germination and elongation under light incubation into a single stage under darkness incubation. These findings modified the protocol and significantly reduced the overall duration of the date palm micropropagation protocol.

Keywords date palm, darkness, germination, protein; phenolics, somatic embryo

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit species in the Middle East and North Africa, it cultivated since ancient times for its delicious and nutritious fruits (Al-Khayri et al. 2018; Jain 2012). In Yemen, many problems are faced the date palm growers. The national program of the date palm is being carried out, aiming at the rehabilitation of the old trees of date palms. Two tissue culture laboratories have been established to produce high-quality date palm cultivars (Ba-Angood 2015).

Somatic embryogenesis is the in vitro propagation process by which bipolar structures, known as somatic embryos, are created from somatic cells without fertilization (Mazri et al. 2020). Developing efficient micropropagation protocols through somatic embryogenesis will participate in the large-scale propagation of elite date palm cultivars (Mazri et al. 2020).

Somatic embryogenesis is the most promising and efficient method for plant micropropagation and a reliable method for studying the morphology and physiology mechanisms of embryo induction and germination (Elhiti et al. 2013; Fehér 2015). To date, all studies and micropropagation protocols of date palm (Phoenix dactylifera) used somatic embryo germination and conversion to plants under light incubation (Almusawi 2015; Fki et al. 2017; Taha 2017; Zayed 2017). The incubation under light gave the highest germination of date palm somatic embryos. While embryos cultured at total darkness gave the lowest values for germination and number of somatic embryos (Shehata et al. 2015). The central focus of research in many palm types is somatic embryo multiplication protocols, but germination and conversion of embryos remain badly studied (Ree and Guerra 2015). Recently only study the effect of light conditions on germination and conversion of indirect date palm somatic embryos to plantlets, the darkness significantly stimulated the conversion of somatic embryos to plantlets. (Abohatem et al. 2020). The effect of light intensity and type on organogenesis has been only studied in date palm (Al-Mayahi 2016; Meziani et al. 2015).

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The incubation conditions on somatic embryo germination have been studied in a few other plant species. Darkness incubation gives an etiolated stem, with fast and continuous apical growth in orchids (Suzuki et al. 2004). On the other hand, light incubation led to inhibited stem longitudinal growth (Suzuki et al. 2010). Darkness incubation *in vitro* was reported to favor somatic embryos in *Curcuma amada Roxb*. (Soundar Raju 2014), in Norway spruce. (Kvaalen and Appelgren 1999), in *Helianthus annuus* L. (Fiore et al. 1997), in *Dendranthema grandiflora Tzvelev*. (May and Trigiano 1991) and in *Cyclamen persicum* Mill. (Takamura and Tanaka 1996).

The main objective of this study is to investigate the effect of light and darkness on the germination and conversion of indirect date palm somatic embryos into plantlets. We compared somatic embryos germinated and converted in light with those germinated and converted in darkness. We also compared the protein content and phenolics of somatic embryos germinated in light with those germinated in darkness.

Material and Method

Preparation and disinfection of plant material

Offshoots from a healthy mother tree of date palm Sultana cultivar from Hadramout Valley are used as the plant material.

The date palm shoot tips were disinfected as described by Abohatem and Baaziz (2015). This method involves soaking shoot tips with a 20% solution of sodium hypochlorite for 30 minutes, then washing three successive with sterile distilled water under a laminar flow hood. The shoot tips were cut into four to six pieces, used as explants, and cultured in the starting medium.

Induction of indirect embryogenic callus

The explants of shoot tip were cultured on a callogenesis induction medium containing Murashige and Skoog (MS) salt (Murashige and Skoog 1962), with 30 g/l sucrose, 150 mg/l activated charcoal, 7 g/l agar, 5 mg/l of 6-Benzylaminopurine (BAP) and 5 mg/l of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Abohatem et al. 2017; Zouine and El Hadrami 2007). For indirect embryogenic callus induction (Fig. 1A), the friable callus formed after 6-8 months of culture was selected and transferred onto a medium containing 0.5 mg/l of BAP and 0.1 mg/l of 2,4-D (Abohatem et al. 2011; El Hadrami and Baaziz 1995). The explants were incubated at 25 ± 2°C in the darkness and subcultured to fresh medium every 5 weeks.

Induction of indirect somatic embryos

For indirect somatic embryo induction (Fig. 1B), the embryogenic callus formed was selected and transferred onto MS medium diluted a half and free of plant growth regulators.

To test the effect of incubation conditions on the induction of somatic embryos a set of 0.2 g embryogenic callus masses were placed in three incubation conditions treatments (16 photoperiods (35 μmol/m2/s of light intensity) provided by cool-white fluorescent lamps, darkness continuous, or darkness for 2 weeks followed by 16 photoperiods) during the period of induction somatic embryos. Cotyledons were counted from mature embryos per treatment.

Germination of the indirect somatic embryos and conversion into plantlets

The mature somatic embryos were selected from the induction medium and transferred onto MS germination medium containing 0.1 mg Naphthalene Acetic Acid (NAA) and 0.15 g activated charcoal (Abohatem et al. 2017).

To test the effect of incubation conditions on the germination of indirect somatic embryos and conversion into plantlets, somatic embryos were placed in three different incubation conditions treatments (16 photoperiod, darkness continuously, or darkness for 2 weeks followed by 16 photoperiod) during the period of germination somatic embryos and conversion into plantlets.

For rooting the plants, the plants formed in all three treatments were transferred into a rooting MS medium free of plant growth regulators under light incubation.

Extraction and analysis of phenolics

The phenolic compounds were extracted and analyzed as described by Macheix et al. (1990). 250 mg fresh tissues of somatic embryos were homogenized with 2 ml of 80%

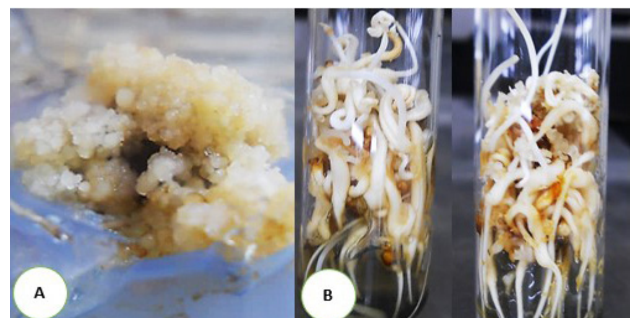

Fig. 1 Induction of embryogenic callus (A) and indirect somatic embryos (B)

methanol at 4°C, centrifuged three times at 7,000 g for 3 min and the supernatants were recuperated each time. 100 µl of the supernatant was added to 250 µl of Folin-Ciocalteu reagent and 20% Sodium carbonate. The mixture was incubated at 40°C for 30 min and the blue colour was measured by spectrophotometer at 760 nm.

Extraction and analysis of proteins

The total proteins were extracted according to the method described by Lecouteux et al. (1993). 250 mg fresh weight of somatic embryo tissues were homogenized with 2 ml Tris maleate buffer (0.1 M, pH 6.5) and centrifuged for 6 min at 7,000 g. Used the supernatant as the crude protein extract. The total proteins were measured by spectrophotometer at 595 nm according to the method described by Bradford (1956).

Statistical analysis

Results were analyzed by variance analysis (ANOVA (followed by SNK test at P = 0.05 level to compare means (SPSS 1996). The number of repetitions is three replicates with two independent experiments.

Results

Effect of incubation conditions on induction of the indirect somatic embryos.

The effects of light and darkness incubation on the induction of the indirect somatic embryos are shown in Table 1.

No significant differences between the numbers of indirect embryos under 16 h photoperiod conditions and the embryos incubated under darkness during the induction of the somatic embryos.

For incubation in the darkness continuously, there was an average of 19 ± 3.2 somatic embryos per 0.2 g fresh weight of callus (Fig. 1B, Table 1) compared with 17 ± 1.9 somatic embryos in the darkness for 2 weeks followed by light and 14 ± 1.7 somatic embryos in the light incubation.

Effect of incubation conditions on germination of indirect somatic embryos and conversion into plantlets

This study has shown that incubation in darkness significantly stimulated germination, elongation and conversion of somatic embryos into plantlets. The somatic embryos that germinated in the darkness exhibited formed of long etiolation stems and fast growth (Fig. 2C, D), while the somatic embryos that germinated in the light showed inhibition of longitudinal growth, with formed short stems and slow growth (Fig. 3A).

The average elongation of indirect somatic embryos germinated, expressed as the length of the embryo stem formed is 13 ± 1.8 cm for incubated in darkness continuously (Fig. 2C, D), 8 ± 0.8 cm for incubated in darkness for 2 weeks followed by 16 h photoperiod (Fig. 2B) and 4 ± 0.4 cm for incubated in 16 h photoperiod (Fig. 3 A) (Table 1).

The average frequency of indirect somatic embryos germinated, expressed as the percentage of germinated embryos formed from the tested embryos is 94 ± 2.3% for incubated in darkness continuously, 90 ± 1.7% for incubated in darkness for 2 weeks followed by 16 h photoperiod and 76 ± 2.6% for incubated in 16 h photoperiod (Table 1).

The average frequency of indirect somatic embryos converted to plantlets expressed as the percentage of plantlets formed from the tested embryos is 92 ± 1.8% for incubated in darkness continuously, 88 ± 1.4% for incubated in darkness for 2 weeks followed by 16 h photoperiod and 73 ± 2.5% for incubated in 16 h photoperiod (Table 1).

Table 1 Effect of incubation conditions on the number of somatic embryos produced, average elongation of embryo stem, frequency of germination, and conversion of indirect somatic embryos into plantlets

Incubation conditions	Average number of somatic embryos per 200 mg FW of embryogenic callus	Average elongation of embryo stem (cm) during conversion from embryo to plantlet	Frequency of germination (%) (germinated/ embryos tested)	Frequency of conversion (%) (plantlets/germinated embryos tested)
16-h photoperiod	14 ± 1.7bc	4 ± 0.4c	76 ± 2.6c	73 ± 2.5c
Continuous darkness	19 ± 3.2a	13 ± 1.8a	94 ± 2.3a	92 ± 1.8a
Two weeks of darkness followed by a 16-h photoperiod	17 ± 1.9ab	8 ± 0.8b	90 ± 1.7ab	88 ± 1.4ab

The values followed by the same letters (a–c) are not significantly different at the p = 0.05 level, as determined by the SNK test.

Fig. 2 Germination and conversion stages of indirect somatic embryos into plantlets under darkness incubation. (A) Indirect somatic embryo stage. (B) Germination and conversion stages of indirect somatic embryos into plantlets under two weeks of continuous darkness incubation, followed by a 16-h photoperiod. (C) Germination and conversion stages of indirect somatic embryos into plantlets under continuous darkness incubation. (D) Conversion stage of indirect somatic embryos into plantlets under continuous darkness incubation following their transfer to incubation under light. (E) Rooting of plantlets derived from somatic embryos converted under darkness

Effect of incubation conditions on micropropagation stages number and time taken during the period of indirect somatic embryo germination and conversion into plantlets

The incubation in darkness significantly decreased the period for conversion of indirect somatic embryos into plants from 24 weeks to 12 weeks (Table 2). The incubation in darkness continuously required only 12 weeks to occur for the conversion of indirect somatic embryos into plants including germination, elongation and rooting plantlets, (Table 2), while the incubation in light required 24 weeks (Table

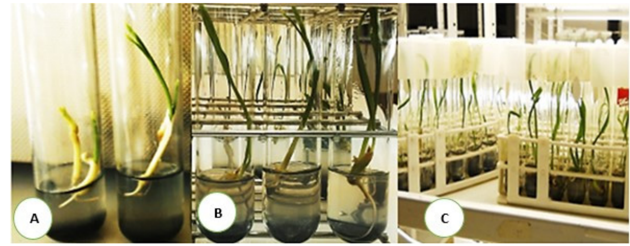

Fig. 3 Germination and conversion stages of indirect somatic embryos into plantlets incubated for a 16-h photoperiod. (A) Germination stage of indirect somatic embryos. (B) Elongation and conversion stages of indirect somatic embryos into plantlets incubated under a 16-h photoperiod. (C) Rooting of plantlets derived from somatic embryos converted under a 16-h photoperiod

2). The incubation in darkness for 2 weeks followed by light required 15 weeks to occur for the germination, elongation and rooting of plantlets (Table 2).

In addition, the incubation in darkness has led to modified date palm micropropagation protocol stages. The incubation in the darkness continuously (Fig. 2C, D) and the incubation in darkness for 2 weeks followed by light led to germination and elongation at one stage (Fig. 2B), leading to a change and decrease in protocol stages, while the incubation in light was required two stages the stage of embryo germination then the elongation stage, and it took longer to form the plants (Table 2, Fig. 3A, B).

Effect of incubation conditions on protein content and phenolics of somatic embryos

There was a significant effect of light and darkness on the protein content of indirect somatic embryos (Table 3). The indirect somatic embryos incubated in the darkness continuously had more protein content (123.46 ± 5.2 μg /g FW) than the embryos incubated in the light (82.67 ± 3.4 μg /g FW) and the embryos incubated in the darkness for 2 weeks

Table 2 Effect of incubation conditions on the number of micropropagation stages and the time required for the germination and conversion of indirect somatic embryos into plantlets

Incubation conditions	Micropropagation stages and duration from indirect somatic embryo germination to plantlet rooting.			
	Stage 1	Stage 2	Stage 3	Total stages
	Time (week)	Time (week)	Time (week)	Total weeks
16-h photoperiod	Germination	Elongation	Rooting	3
	8 ± 0.5a	8 ± 0.2	8 ± 0	24 ± 0.7a
Continuous darkness	Germination and elongation	Rooting	----------	2
	4 ± 0.2c	8 ± 0	-----------	12 ± 0.2c
Two weeks of darkness followed by a 16-h photoperiod	Germination and elongation	Rooting		2
	7 ± 0.3ab	8 ± 0	-----------	15 ± 0.3b

The values followed by the same letters (a–c) are not significantly different at the p = 0.05 level, as determined by the SNK test.

Table 3 Effect of incubation conditions on proteins and phenolic compound content in indirect somatic embryos

Incubation conditions	Total protein μg/g FW	Phenolic compounds mg/g FW
16-h photoperiod	82.67 ± 3.4c	0.28 ± 0.06a
Continuous darkness	123.46 ± 5.2a	0.22 ±0.08bc
Two weeks of darkness followed by a 16-h photoperiod	113.34 ± 4.5b	0.25 ± 0.04ab

The values represent means ± standard error, derived from three replicates across two experiments. The values followed by the same letters (a-c) are not significantly different at the p = 0.05 level, as determined by the SNK test.

followed by light (113.34 ± 4.5 μg /g FW) (Table 3). The indirect somatic embryos incubated in the light had a higher concentration of phenolics (0.28 ± 0.06 mg / g FW) than the embryos incubated in the darkness continuously (0.22 ±0.08 mg / g FW) and the embryos incubated in the darkness for 2 weeks followed by light (0.25 ± 0.04 mg / g FW) (Table 3).

Discussion

This study has shown that incubation in darkness significantly stimulated germination and conversion of somatic embryos into plantlets. These results contrast with all studies on date palm somatic embryogenesis, which used incubation of germination somatic embryos and conversion into plants under light, and neglected the germination under the darkness incubation that mimics the germination of the zygotic embryo in the seed. So, these findings contribute new information to understanding light's influence on the germination of in vitro somatic embryos.

Our hypothesis is that date palm somatic embryo germination is similar to the germination of zygotic embryos. Date palm somatic embryo conversion is similar to the germination of zygotic embryos (Mazri and Mezian 2015; Ree and Guerra 2015). Success of the somatic embryo germination depends on the formation of both the radicle and plumule and the reserve compounds accumulation enough to grow plantlet until it can boost itself by photosynthesis (Bekheet et al. 2001, Ree and Guerra 2015). The date palm zygotic embryo germinates in darkness and its growth is dependent on the nutritional compounds stored in the seed. This germination process is similar to somatic embryo germination in the darkness, where the germination of the somatic embryos does not need light and their growth in vitro is primarily dependent on the sugar and the compounds added to the culture medium, which leads to germination and elongation of somatic embryos (Ferreira et al. 2011). Our results showed clearly the importance of darkness (i.e., etiolation) on germination and conversion of date palm somatic embryos into plantlets. Our results are in agreement with previous reports on a few plant species such as *Prunus incisa* (Cheong and Pooler 2004), Sengon (*Falcataria moluccana*) (Sunandar et al. 2017), *Dendrobium Second Love* (Ferreira et al. 2011), and *Cyathea delgadii Sternb* (Mikuła et al. 2015).

The study has shown that the indirect somatic embryos incubated in the darkness formed etiolation long stems and fast growth (Fig. 2B, C, D), while the somatic embryos incubated in the light formed short stems and slow growth (Fig. 3A). Similar results were obtained in other plants, such as *Catasetum Fimbriatum* (Suzuki and Kerbauy 1999, 2006; Suzuki et al. 2010).

The positive effect of darkness on stimulation germination and elongation of indirect somatic embryos was due to an increase in the hormone levels and sugars soluble in the darkness incubation during embryogenesis (Grzyb et al. 2017). By changes in the photoperiod conditions could be organized by phytohormone concentrations and their signaling pathways (Pacholczak et al. 2005; Symons and Reid 2003). Incubation in darkness gives a signaling cue for cells to decide to go in the growth direction, which is caused by increasing the endogenous hormone level (Zobayet and Saxena 2003), and significantly decreasing the concentration of abscisic acid (ABA) (Grzyb et al. 2017). Light induces IAA-peroxidase activity, which causes the breaking down of IAA (Liu et al. 1996). The gibberellin has a relationship with light in establishing etiolated growth and the repression of photomorphogenesis (Alabadí et al. 2004).

In this study, the incubation in darkness led to a significantly decreased period for conversion of indirect somatic embryos to plants from 24 weeks to 12 weeks. All protocols of date palm micropropagation determined that somatic embryo germination and conversion into plants occurred under light and required about 24 weeks period from germination until the rooting stage (Al-Khalifah et al. 2013). So, this result is like a breakthrough in developing date palm micropropagation by decreasing the protocol period by more than 3 months.

The positive effect of darkness incubation on decreasing the protocol period by more than 3 months is due to an essential characteristic associated with somatic embryo

germination in the darkness or light, which is closely related to the shoot tip meristem activity (Griffiths and Halliday 2011). When incubated in the darkness a dramatic elongation of the stem, characterized by fast and continuous shoot tip longitudinal growth, that resulted from cells multiplication. On the other hand, when incubated in the light they show inhibition of longitudinal growth, which resulted from the effect of light on the suppression of the mitotic activity of shoot tip meristem, which could be characterized as a dormancy condition (Suzuki and Kerbauy 2006; Suzuki et al. 2010).

Furthermore, the incubation in the darkness led to germination and elongation at one stage (Table 2, Fig. 2B, C, D), leading to a change and decrease of protocol stages and decreased protocol period, while the incubation in the light was required two stages the embryo germination stage then the elongation stage and it took longer time to formation the plants (Table 2, Fig. 3A, B). The previous protocols determined the embryo germination stage and then the elongation stage (Abohatem et al. 2017; Al-Khalifah and Shanavaskhan 2012).

This study showed that the somatic embryos incubated in the darkness had significantly more protein content and low phenolics than those in the light (Table 3). In many reports, darkness treatment was reported to decrease the production of phenolic compounds than in light (Aderkas et al. 2015; George 1993) which was caused by inhibition of an oxidation enzyme in plant tissue (Zobayet and Saxena 2003). These results gave new important information to our understanding of the effect of darkness on the accumulation of storage products because the germination of somatic embryos depends on the accumulation of these compounds enough to grow into plantlets. The high accumulation of protein in the darkness in our experiment is an important reason why somatic embryos germinate easily and convert fully to plantlets. The high storage capacity of protein and sugar in somatic embryos incubated in the darkness showed the importance of providing nutritional storage to support germinating somatic embryos and converting them to plantlets. (Aderkas et al. 2015; Grzyb et al. 2017).

Conclusion

Our study offers a new interpretation of the role of darkness on somatic embryo germination and conversion into plantlets, as mimics the same germination of zygotic embryos in the seed. These results confirmed the efficient role of darkness incubation in stimulating the germination of somatic embryos and conversion into plantlets. The high accumulation of protein in the darkness is an important reason why somatic embryos germinate easily and convert fully to plantlets. It has led to a decrease in the protocol period and modified the date palm micropropagation protocol stages.

Author Contributions

AM performed and designed experiments, analyzed and interpreted data and wrote the manuscript. AY performed biochemical data analysis. AH contributed to the writing and editing of the tables and figures.

Conflict of Interest

The authors declared that there is no conflict of interest.

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