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# INDUCTION OF SOMATIC EMBRYOS AND IMPROVEMENT OF EMBRYOGENIC CALLUS BY TWO TYPES OF SILVER ON DATE PALM (*PHOENIX DACTYLEFERA* L.)

[19]

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**Keywords:** Somatic embryos, Silver type, Date palm, Proliferation

## ABSTRACT

The response of embryogenic callus of date palm cvs. (Pertomuda and Skooty) was tested on media containing two types of silver (STS and $AgNO_3$) alone or combined with 0.25 & 0.5 mg/l 2ip. Addition of STS onto callus induction medium significantly enhanced embryogenic callus production (both growth and induction). $AgNO_3$ as a heavy metal ion made a stress treatment on callus to proliferation embryos than STS, this proliferation was increased at a low concentration of $AgNO_3$, but in the presence of 2ip gave the opposite trend, the embryos increased at 4 mg/l or 6 mg/l $AgNO_3$ + 0.5 mg/l 2ip and proceeded normally embryos for both Pertomuda and Skooty date palm.

**Abbreviations:** STS = Silver thiosulphate; EC = embryogenic callus; NAA = α-naphthaleneacetic acid and 2ip = N6-2-isopentenyladenine.

## INTRODUCTION

The induction and regeneration of somatic embryos are very sensitive to culture conditions such as the composition of the medium, the physical environment of the medium, the culture, the genotype and the explants source **(Fuentes *et al* 2000)**. The environment of ethylene in plant tissue (growth and differentiation) had been widely investigated. Application of ethylene precursors and/or inhibitors has shown that ethylene may often have divers effects on similar tissue culture systems. Although it has been reported that ethylene may promote callus growth **(Songstad *et al* 1991)**, it generally appears to inhibit somatic embryogenesis and shoot regeneration **(Biddington, 1992)**. Silver nitrate ($AgNO_3$), is a potent inhibitor of ethylene action **(Beyer, 1976)**, stimulated somatic embryo development in carrot **(Roustan *et al* 1994)**.

The most effective silver type is STS (silver thiosulphate) as 1.8 or 1.4 complex of silver nitrate and sodium thiosulphate available as Argylene (1 g/l = 0.2 mM $Ag^+$) **(Fjeld and Moe, 1985)**. The STS complex was used because it has much greater mobility in plant tissue than uncomplexed silver ion **(Veen and Van De Geun, 1978)**. **Mollers *et al* 1992** found that the addition of silver thiosulphate to *In Vitro* potato shoot propagation at 1.5 mg/l led to an increase of leaf material. Also, **Le (1990)** indicated that, the addition of silver thiosulphate to the culture medium improved the explants growth of potato cvs.

Besides functioning as an ethylene-action inhibitor, $AgNO_3$ may serve as a stress agent. The successful use of heavy metal ions to induce somatic embryo formation on the surface of carrot seedlings as they act as a stress treatment **(Kamada *et al* 1989, Kiyosue *et al* 1990)**. Since stress induces endogenous ABA accumulation **(Walker-Simmons and Sesing, 1990, Hauser *et al* 1992)**, $Ag^+$ being a metallic ion may also promote somatic embryo production via an increase in the endogenous ABA levels.

In date palm (*Phoenix dactylifera* L.) callus proliferation that normally occurs prior to redifferentiation upon callus transfer to hormone-free regeneration medium, as well as subsequent somatic embryogenesis, were shown to be stimulated by $AgNO_3$ added to the regeneration medium in cv. Barhee **(Al-Khayri and Al-Bahrany, 2001)**.

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The objectives of the present study were to test the effect of two types of silver on enhancing the production of embryogenic callus in two date palm cvs. And to induce the highest number of somatic embryos.

## MATERIALS AND METHODS

### 1- Establishment of callus induction

Shoot tips of date palm (*Phoenix dactylefera* L.) pertomuda and skooty cvs. were excised and cultured on MS medium **(Murashige and Skoog, 1962)** supplemented with (per liter) 170mg $NaH_2PO_4$, 100 mg Myo-Inositol, 200mg glutamine, 2 mg glycine, 1mg biotin, 1mg thiamine-HCl, 1mg nicotinic acid, 1mg pyridoxine-HCl, 1mg calcium pantothenate, 30g sucrose, 7g agar (phytochnology), 100mg 2,4-dichlorophenoxyacetic acid (2,4-D), 3 mg 2-isopentenyladenine (2ip) and 3 g activated charcoal. These cultures were maintained in darkness at 27± 2°C for 6 months. The resultant callus was transferred to callus proliferation medium that contained MS salts augmented with 10 mg/l 2,4-D and 1.5 g/l activated charcoal for 2 months in darkness at 27± 2°C for embryogenic callus (EC) formation. Media were adjusted to pH 5.7 and autoclaved for 15 min at 121 °C and 1.1 $Kg/cm^{-2}$.

### 2- Influence of silver type alone and combined with 2ip.

To evaluate the type of silver alone or combined with 2ip on maximum embryogenic callus (EC) fresh weight. The explants was cultured on nutrient medium supplemented with different concentration of silver thiosulphate (STS) or $AgNO_3$ at 2, 4, 6 mg/l and combined with 0.25 or 0.5 mg/l 2ip. Each small jar (150 $cm^3$) was inoculated with 0.30 embryogenic callus. The cultures were kept in darkness at 25± 2°C after 2 months the EC fresh weight was measured which transferred every 4 weeks to new culture (two subcultures).

After that, EC was transferred to MS medium supplemented with 0.1 mg/l Naphthaleneacetic acid (NAA), 30 g/l sucrose, 7 g/l agar, 1 mg/l thiamin, 1 mg/l Nicotinic, 1 mg/l Pyridoxin. HCl, 1 mg/l Biotin and the same concentrations of silver (STS or $AgNO_3$) and combined with 0.25 and 0.5 mg/l 2ip to induce somatic embryos. Cultures were maintained at 25± 2°C and 16 h photoperiod. The number of embryos per culture was counted after two subcultures at 6 weeks intervals.

### 3- Statistics

Nine jars were used for each tested concentration containing (0.3 g callus). Factorial Randomized Complete Black Design was used and data were subjected to analysis of variance separation of means among treatments was determined using LSD test at 5% according to **Steel and Torrie (1980)**.

## 4- RESULTS

### 4.1. The effect of silver concentration on callus fresh weight and number of somatic embryos.

#### 4.1.1. cv. Pertomuda

Callus growth, expressed in fresh weight of cv. Pertomuda was significantly increased in response to adding of STS or $AgNO_3$ to the callus medium **(Table 1)**. STS produced the highest mean weight of callus (0.99 g) compared with $AgNO_3$ (0.29 g). Further increase of STS stimulated gradual increase in callus fresh weight, reaching the maximum at 6.0 mg/l STS.

Silver increased embryo production at low concentration after light exposure **(Table 1)**. $AgNO_3$ was more effective than STS to induce embryos, addition of $AgNO_3$ to nutrient medium gave 7.44 embryos/cultures. The highest number of embryos was obtained at 2.0 or 4.0 mg/l $AgNO_3$ or STS (7.49 & 5.49) without significant differences between them, but the higher concentrations of silver, caused a reduction in embryo number.

#### 4.1.2. cv. Skooty

The effect of adding silver to callus induction medium on culture of date palm cv. Skooty was shown in **Table (2)**. The presence of silver showed remarkable effect for callus growth. The highest mean of callus fresh weight was observed from STS silver type, it increased significantly callus fresh weight (1.32 g) than $AgNO_3$ (0.50 g). There were no significant differences among the three silver concentrations on callus fresh weight, in this regard, 6.0 mg/l STS or $AgNO_3$ gave the maximum weight of callus.

Addition of both STS or $AgNO_3$ to nutrient medium was effective to induce somatic embryos and did not cause an inhibition of embryos development, $AgNO_3$ induced the highest number of somatic embryos culture (5.77) in the 16 h light.

**Table 1. Induction of somatic embryos by two silver types of date palm cv. Pertomuda**

| Conc. of Silver (mg/l) | Callus fresh weight (g) | | | No. of embryos | | |
|---|---|---|---|---|---|---|
| | STS | $AgNO_3$ | Mean (B) | STS | $AgNO_3$ | Mean (B) |
| 2.0 | 0.86 | 0.26 | 0.56 | 4.33 | 10.66 | 7.49 |
| 4.0 | 0.86 | 0.30 | 0.58 | 3.33 | 7.66 | 5.49 |
| 6.0 | 1.26 | 0.33 | 0.79 | 3.33 | 4.00 | 3.66 |
| Mean (A) | 0.99 | 0.29 | | 3.66 | 7.44 | |

| L S D at 0.05 | | |
|---|---|---|
| A | 0.17 | 2.21 |
| B | NS | 2.71 |
| AB | NS | NS |

**Table 2. Induction of somatic embryos by two silver types of date palm cv. Skooty**

| Conc. of Silver (mg/l) | Callus fresh weight (g) | | | No. of embryo | | |
|---|---|---|---|---|---|---|
| | STS | $AgNO_3$ | Mean (B) | STS | $AgNO_3$ | Mean (B) |
| 2.0 | 1.4 | 0.56 | 0.98 | 5.00 | 8.00 | 6.50 |
| 4.0 | 1.06 | 0.46 | 0.76 | 3.66 | 5.66 | 4.66 |
| 6.0 | 1.50 | 0.50 | 1.00 | 3.66 | 3.66 | 3.66 |
| Mean (A) | 1.32 | 0.50 | | 4.10 | 5.77 | |

| L S D at 0.05 | | |
|---|---|---|
| A | 0.20 | 1.11 |
| B | NS | 1.36 |
| AB | NS | NS |

The minimum silver concentration induced the highest significant embryo number (6.50) while, the maximum concentration produced the lowest number.

Although, silver had a pronounced influence on the process of somatic embryogenesis, the germination of the resultant somatic embryos and their development into plantlet (data untabulated).

**5. The effect of silver type combination with 2ip.**

**5.1. Callus fresh weight of cv. Pertomuda**

Results in **Table (3)** show the effect of silver type in the presence of 2ip on callus induction medium.

The callus fresh weight was greater with STS (0.76 g) than $AgNO_3$ (0.20 g), the two tested 2ip concentrations enhanced callus fresh weight.

**5.2. Number of embryos of cv. Pertomuda**

Differences were observed between the two types of silver on number of embryos **Table (3)**. $AgNO_3$ had the highest mean number of embryos (2.22), while STS, only showed lower number (1.49) embryos. Addition of 2ip with silver enhanced the formation of somatic embryos of date palm, 0.5 mg/l 2ip gave the highest number of embryos. $AgNO_3$ at 6 mg/l combined with 0.5 mg/l 2ip increased embryo induction and the germination proceeded normally.

**Table 3. Effect of silver type and 2ip on date palm cv. Pertomuda**

| Type of silver (A) | Conc. of silver mg/l (B) | Conc. of 2ip (mg/l) (C) | Callus fresh weight (g) | No. of embryos |
|---|---|---|---|---|
| STS | 2 | 0.25 | 1.00 | 2.00 |
| | | 0.50 | 0.66 | 1.66 |
| | 4 | 0.25 | 0.90 | 3.00 |
| | | 0.50 | 0.56 | 1.00 |
| | 6 | 0.25 | 0.73 | 0.66 |
| | | 0.50 | 0.73 | 0.66 |
| **Mean A** | | | 0.76 | 1.49 |
| $AgNO_3$ | 2 | 0.25 | 0.10 | 1.00 |
| | | 0.50 | 0.26 | 2.66 |
| | 4 | 0.25 | 0.16 | 1.00 |
| | | 0.50 | 0.30 | 3.33 |
| | 6 | 0.25 | 0.13 | 0.00 |
| | | 0.50 | 0.26 | 5.33 |
| **Mean (A)** | | | 0.20 | 2.22 |
| **Mean (B)** | | | 0.50 0.48 0.46 | 1.83 2.08 1.66 |
| **Mean (C)** | | | 0.50 0.46 | 1.27 2.44 |

L S D at 0.05

| | | |
|---|---|---|
| A | 0.11 | 0.45 |
| B | NS | NS |
| AB | NS | 0.78 |
| BC | NS | 0.78 |
| ABC | NS | 1.10 |

### 5.3. Callus fresh weight of cv. Skooty

After 8 weeks of culture on nutrient medium supplemented with different concentrations of silver combined with 2ip, different response could be observed in **(Table 4 and Fig. 1)**. STS promotes callus production (both growth and induction). All concentrations of silver (STS, $AgNO_3$) enhanced callus fresh weight.

### 5.4. Number of embryos of cv. Skooty

As for the response of silver, addition of both STS or $AgNO_3$ to the nutrient medium was effective to induce somatic embryos and did not cause any inhibition to embryo development **(Table 4 and Fig. 1)** the optimum concentration increased somatic embryos was 4.0 mg/l silver. Number of embryos was significantly influenced by the interaction between silver and 2ip, the number of resultant embryos was highest on 4 mg/l $AgNO_3$ with 0.5 mg/l 2ip, the action of silver was clearly modified by addition of 2ip, because 2ip forms embryos without vitrification.

Generally, the result mentioned that, the maximum silver concentration of STS gave the highest callus fresh weight, while the minimum silver concentration of $AgNO_3$ induced the highest somatic embryos for the two cvs. Silver combined with 2ip increased embryogenesis without vitrification and embryos developed normally.

## DISCUSSION

Silver appears unique among heavy metals as an inhibitor of ethylene action **(Beyer, 1976)**. Transferring embryogenic callus of the different date palm to different concentrations of silver to induce somatic embryos was the aim of this study.

**Table 4. Effect of silver type combination with 2ip of date palm cv. Skooty**

| Type of silver (A) | Conc. of silver mg/l (B) | Conc. of 2ip (mg/l) (C) | Callus fresh weight (g) | No. of embryos |
|---|---|---|---|---|
| STS | 2 | 0.25 | 1.3 | 2.66 |
| | | 0.50 | 0.76 | 2.00 |
| | 4 | 0.25 | 1.03 | 3.00 |
| | | 0.50 | 0.70 | 1.33 |
| | 6 | 0.25 | 0.9 | 0.66 |
| | | 0.50 | 0.86 | 0.66 |
| **Mean A** | | | 0.92 | 1.71 |
| AgNO3 | 2 | 0.25 | 0.16 | 0.00 |
| | | 0.50 | 0.56 | 2.33 |
| | 4 | 0.25 | 0.33 | 1.33 |
| | | 0.50 | 0.56 | 3.33 |
| | 6 | 0.25 | 0.20 | 0.00 |
| | | 0.50 | 0.36 | 3.66 |
| **Mean (A)** | | | 0.36 | 1.77 |
| **Mean (B)** | | | 0.69 / 0.65 / 0.58 | 1.47 / 2.24 / 1.24 |
| **Mean (C)** | | | 0.65 / 0.63 | 1.27 / 2.21 |

L. S. D. at 0.05

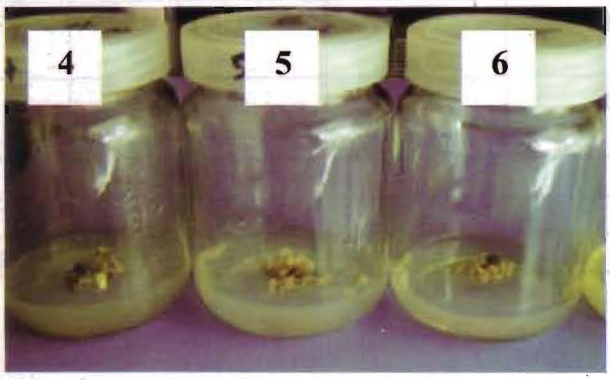
| | | |
|---|---|---|
| A | 0.16 | NS |
| B | NS | 0.64 |
| AB | NS | 0.90 |
| BC | NS | 0.90 |
| ABC | NS | NS |

The current study observed that, $AgNO_3$ as a heavy metal ion made a stress treatment on callus to proliferate embryos than STS, this proliferation was increased at a low concentrations of $AgNO_3$. **Biddington *et al* 1988** suggests that, $AgNO_3$ promotes embryogenesis in *Brussels sprouts* by blocking the inhibitory effect of endogenous ethylene on embryo production. It is also possible that, stimulation by $AgNO_3$ in white spruce is gradually eclipsed by higher concentrations of exogenous ABA in the maturation medium combined with the results of ABA analysis, demonstrate that $AgNO_3$ may affect embryo maturation mainly by influencing endogenous ABA levels **(Kong and Yeung, 1995)**. In carrot, $AgNO_3$ can stimulate somatic embryo formation even in the presence of an exogenous ethylene source, ethephon **(Roustan *et al* 1990)**. **Kamada *et al* (1993)** found that, gene expression of a protein (ECP31) directly related to embryogenic competence, was commonly observed in some stress treatments which could induce somatic embryogenesis in carrot. The gene expression of ECP 31 was also controlled by abscisic acid.

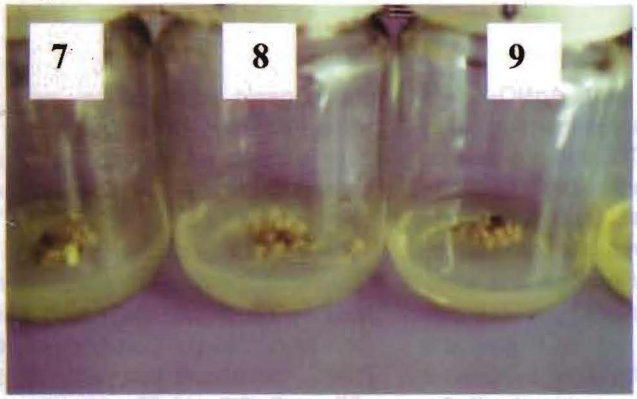
Silver at higher levels caused a reduction on number of somatic embryos. This result agree with **Biddington *et al* (1988) and Al-Khayri and Al-Bahrany (2004)**. Conversely, **Hatanaka *et al* (1995)** mentioned that addition of $AgNO_3$ (5-50 µM) or CoCl2 (10-50 µM) throughout all culture stages inhibited the formation of somatic embryos in leaf explants of *Coffea canephora*.

The STS complex was used because it has much greater mobility in plant tissue than uncomplexed silver ion **(Veen and Van De Geun, 1978)**. In this study, adding silver to callus induction medium enhanced the growth of callus, STS at 6 mg/l was the most effective concentration for increasing


1, 2, 3= 2, 4, 6 mg/l AgNO3 concentrations




4, 5, 6 = 2mg/l $AgNO_3$+0.25 2ip, 2mg/l $AgNO_3$ +0.5 2ip, 4mg/l $AgNO_3$ +0.25 2ip concentrations




7, 8, 9 = 4mg/l $AgNO_3$+0.5 2ip, 6mg/l $AgNO_3$+0.25 2ip, 6mg/l $AgNO_3$+0.5 2ip concentrations

**Fig. 1. Effect of different concentration of $AgNO_3$ combined with 2ip to induce somatic embryos of date palm cv. Skooty**

callus fresh weight, however, STS was producing the highest weight of callus for both cvs.. Similarly, the most effective silver type is $Ag^+$ as (STS) a 1:8 or 1:4 complex of silver nitrate and sodium thiosulphate available as argylene (1 g/l = 0.2 mM $Ag^+$) **(Fjeld and Moe, 1985)**. **Balletti *et al* (1994)** suggested that the addition of silver thiosulphate (STS) to the culture medium improved the growth of potato explant cvs..

In date palm, the number of embryos increased in response to increasing silver nitrate concentration in the absence of 2ip. In the presence of 2ip, the number of resultant embryos was high on 25 micro M $AgNO_3$ in the combination of 0.5 micro M 2ip, callus growth gradually increased as the $AgNO_3$ concentration increased **(Al-Khayri and Al-Bahrany, 2001; 2004)**. Callus initiation rate was improved when immature embryos were cultured on a modified Murashige & Skoog medium containing various silver nitrate concentrations **(Vain *et al* 1989)**.

In contrast to our results, **Tsao and Reed, 2002** reported that silver nitrate significantly reduced callus growth in *Rubus* spp. a 59 µM and in *Saccharum spp.* at 29-118 µM **(Taylor *et al* 1994)**.

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# تحفيز انتاج الاجنة الجسدية وتحسين الكالوس الجنيني بواسطة نوعين من الفضة على نخيل البلح

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**الملخص العربى**

يهدف هذا البحث الى دراســة تــاثير ثيوســلفات الفضة ونترات الفــضة وحــدها او مــع تركيــزات ٠,٢٥، ٠,٥٠ مليجرام / للتر من الايزوبنتيل ادينــين (2ip) على الكالوس الجنيني لنخيل الــبلح لاصــناف البرتمودا و السكوتي .

اوضحت النتائج ان اضافة ثيوسلفات الفضة الى بيئة نمو الكالوس يؤدي الى زيادة انتــاج الكــالوس الجنيني من حيث النمو والنضج.

تعتبر الفضة من العناصر الثقيلــة والتي تــؤدي الى عمل اجهاد في البيئة. وهذا أدى إلــــى زيــادة في تخليق الاجنــة بعــدد اكبر منــه فــى حالــــة استخدام مادة ثيوسلفــات الفضة وهــذه الزياده فــي الاجنه تحدث عنــد التركيزات المنخفضة ، ولكن في وجود الايزوبنتيل ادينــين (2ip) يحــدث العكــس ، ويزداد تركيز نترات الفضه الى اربعه مليجرام / للتر + ٠,٥ مليجرام / للتر 2ip وتنمو الاجنة طبيعيا .

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