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Somatic embryogenesis and plant regeneration from shoot-tip explants in *Phoenix dactylifera* L.

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Abstract

For maximum avoidance of somaclonal variation risks, the commonly used medium for somatic embryogenesis in *Phoenix dactylifera* has been lowered in growth regulators and activated charcoal. When initially cultured on MS basal medium containing only 150 mg dm^{-3} charcoal, 5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 mg dm^{-3} benzylaminopurine (BAP), 10 to 20 % of shoot-tip explants developed into embryogenic calli. The embryogenic potential has been maintained for over 24 months with no decline. In addition, this medium has been found to be more efficient than conventionaly one containing 3 g dm^{-3} charcoal, 100 mg dm^{-3} 2,4-D and 3 mg dm^{-3} 2-isopentyladenosine (2IP). Plantlet regeneration was achieved when somatic embryos were subcultured to medium with 0.1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} BAP or without growth regulators.

Key words: benzylaminopurine, charcoal, date palm, 2,4-dichlorophenoxylacetic acid, 2-isopentyladenosine, tissue browning

Introduction

The date palm (*Phoenix dactylifera* L.), a perennial allogamous monocotyledon, has a great value particularly in North African area by its economical importance and environmental impact. This system has been propagated for a long time by separating the vegetative offshoots from a parent plant. Such conventional method is limited by the number of offshoots produced during the life of a parent plant. In addition, this method is actually one of the factors that propagate Bayoud, a vascular disease, in date palm growing regions of the world. The tissue culture of date palm has been

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reported (Tisserat 1979, Sharma *et al.* 1984, Mater 1986, Zaid 1989) but plant regeneration through somatic embryogenesis is largely limited. At the present numerous date palm cultivars are considered as recalcitrant systems with regard to this method. The tissue browning constitute one among other factors that limit the obtention of somatic embryos (El Hadrami and Baaziz 1994). This phenomenon has also been reported in other species like *Parthenium argentatum* (Trautman and Visser 1989), *Fagus sylvatica*, *Quercus petraea*, *Coffea* (Bonga and Durzan 1987) and *Hevea brasiliensis* (El Hadrami and d'Auzac 1992, Housti *et al.* 1991). Several factors have been added to media with the aim to limit tissue browning such as ascorbic acid, polyvinylpyrrolidone and activated charcoal (Bonga and Durzan 1987). In date palm, it has been described that activated charcoal was essential for somatic embryogenesis (Tisserat 1982). Its use at 3 g dm^{-3} imposed the administration of a high concentration: 100 mg dm^{-3} of 2,4-dichlorophenoxyacetic acid (2,4-D) in the commonly used medium. Such conditions have been recently reported as the best one for obtaining embryogenic calli (Letouze and Daguin 1989).

For maximum avoidance of somaclonal variation risks, we report in this communication the first establishment and multiplication of date palm regenerable embryogenic cultures from shoot-tip explants in a medium twenty times lowered in 2,4-D and charcoal.

Materials and methods

Explants derived from young offshoots of two date palm cultivars (Iklane and Jihel) were maintained in culture to induce callus formation at 26 ± 2 °C in the dark. The culture was subcultured every 6 weeks on the medium described by Tisserat (1979) and Bhaskaran and Smith (1992). This medium is commonly supplemented with 100 mg dm^{-3} 2,4-D, 3 mg dm^{-3} 2-isopentyl-adenosine (2IP) and 3 g dm^{-3} activated charcoal. After preliminary studies, this medium (MD1) was compared to the same one containing only 5 mg dm^{-3} 2,4-D, 0.15 mg dm^{-3} 2IP and 150 mg dm^{-3} charcoal (MD2) and 5 mg dm^{-3} 2,4-D, 5 mg dm^{-3} benzylaminopurine (BAP) and 150 mg dm^{-3} charcoal (MD3). After 4 months, the calli were transfered to fresh medium containing 0.1 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} BAP and 150 mg dm^{-3} activated charcoal under the same culture conditions as before. In this medium, embryogenic calli and somatic embryos were developed within 2 to 4 months.

Results

Shoot-tip explants cultured on three culture media containing different hormone compositions produced calluses, in general, within 4 - 10 weeks. After 80 d of culture, callus induction was obtained with 90 % of explants cultured in the commonly used medium (MD1). In addition, tissue browning was decreased in this medium in both date palm cultivars (Iklane and Jihel, Table 1). In comparison with MD1, the media MD2 and MD3 have been largely lowered in plant growth

regulators and charcoal. MD2 which contained only 5 mg dm^{-3} 2,4-D, 0.15 mg dm^{-3} 2IP and 150 mg dm^{-3} charcoal was not favourable for callus induction (explant reactivity) and led, for each cultivar, to a maximum of tissue and medium browning. After preliminary experiments (data not shown), when the ratio auxin/cytokinin was equal to one and particularly when 2IP was substituted by BAP, the callogenesis induction was enhanced and tissue browning was reduced (Table 1). This latter result prompted us to investigate the possibilities to obtain embryogenic calli in hypoauxinic media in comparison with the routine procedure where the calli have been subcultured on the same medium MD1 every 30 - 40 d until the formation of embryogenic callus that required at least 12 months.

Table 1. Comparison of callogenesis, tissue browning and percentage of explant reactivity (start of callogenesis) in two date palm cultivars in three culture media after 80 d of culture. For each treatment 60 to 80 explants were used. (++ - important, +++ - very important, + - not very important)

Cultivars		Medium MD1	MD2	MD3
Iklane	callogenesis	+++	++	+++
	tissue browning	++	+++	++
	explant reactivity [%]	90	73	89
Jihel	callogenesis	+(+)	++	++
	tissue browning	+	++(+)	++
	explant reactivity [%]	90	75	92

Table 2. Percentage of embryogenic calli obtained after 6 - 8 months of culture in three culture media in cv. Iklane and cv. Jihel. (In parentheses number of embryogenic calli/number of subcultured calli.)

Cultivars	Embryogenic calli [%]		
	Medium MD1	MD2	MD3
Iklane	4 (2/50)	4 (2/51)	20 (12/60)
Jihel	0 (0/62)	0 (0/65)	10 (5/50)

In general, the percentage of responding shoot-tip explants ranged from none in cv. Jihel to 0 - 5 % of embryogenic calli in cv. Iklane with the standard procedure. In these experiments, MD1 and MD2 media have lead to 0 % of embryogenic calli (white, friable and nodular calli) (Fig. 1a,b) in cv. Jihel and to 4 % in cv. Iklane. MD3 medium turned out to be slightly better than MD1 and MD2. It leads to 10 % and 20 % of embryogenic calli in cv. Jihel and cv. Iklane respectively (Table 2). When transfered to medium with 0.1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} BAP, the embryogenic masses showed a high rate of multiplication (Fig. 1b) and developed into regenerating calli with embryos at different stage of development (Fig. 1b,c). The initial fresh mass increased about 4 fold within 60 to 80 d of culture (Fig. 1b).

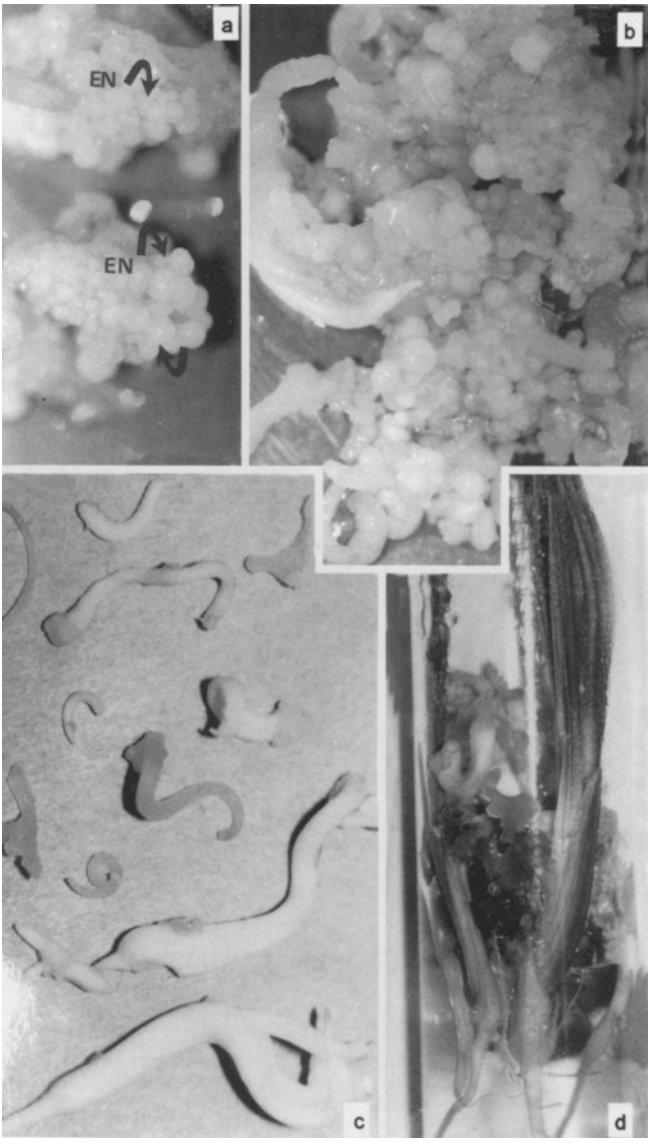

Fig. 1a. Examples of date palm white, nodular and friable embryogenic calli with embryogenic nodules (EN, arrows).

Fig. 1b. Light microscopy of embryogenic callus showing a high rate of multiplication on the new culture medium developed (MD3) containing 0.1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} BAP.

Fig. 1c. Light microscopy of various stages of development in date palm somatic embryos and first stage of plantlet development.

Fig. 1d. Shoot and root regeneration. Somatic embryos have been cultured on medium MD3 with or without growth regulators.

In this medium, the embryogenic potential has been maintained for over 24 months with no decline. Plantlet regeneration was achieved in this later medium or in the same without growth regulators (Fig. 1d).

Discussion

The data reported here shows that exogenous supply of activated charcoal (3 g dm^{-3} or only 150 mg dm^{-3}) limits tissue browning in date palm. Similar results have already been described (Gabr and Tisserat 1985) and also for other plants *e.g. Fagus sylvatica* and *Quercus petraea* (Bonga and Durzan 1987). Nevertheless, this preventive effect was not systematically related to somatic embryogenesis initiation. In some cases, embryogenic potential was lowered at least at the beginning of culture by charcoal in date palm (results not shown). According to Vieitez *et al.* (1992), browning of tissues which constitute a problem in micropropagation did not impair the embryogenic capacity. On MD1 medium, the lowered embryogenic competence could be related to the influence of other factors particularly plant growth regulators that must be adsorbed by charcoal. Such a situation could perturbate the auxin/cytokinin ratio and acts as a potential inhibitor of somatic embryogenesis.

Higher concentrations of 2,4-D have been mostly used for callogenesis and somatic embryogenesis initiation in *Elaeis guineensis* (Touchet *et al.* 1991), *Cocos nucifera* (Buffard-Morel *et al.* 1992) and *Phoenix dactylifera* (Bhaskaran and Smith 1992). Such dependence of somatic embryogenesis induction on 2,4-D level was observed in other monocotyledons (Radojevic 1988). In date palm, the establishment of embryogenic cultures from calli originating from MD1 medium with 100 mg dm^{-3} 2,4-D required at least 12 months. Similar results have been recently reported in date palm cv. Barhee (Bhaskaran and Smith 1992). However, the percentage of these regenerating calli was, in general, relatively low (about 5 %) with the standard procedure. The relative recalcitrance especially with cv. Jihel may be explained by the excess of plant growth regulators at the start of culture and by the long time contact of tissue with this high level. This inhibition with exogenous hormones has been clearly described in *Hevea brasiliensis* (El Hadrami *et al.* 1991, El Hadrami and D'Auzac 1992). The genotypic differences between cvs. Jihel and Iklane in the endogenous hormone contents of the explant, and in the accumulation and sensitivity to these hormones could explain the genotypic variations of embryogenic potential. These effects have been discussed by Carman *et al.* (1988), Wenk *et al.* (1988) and El Hadrami *et al.* (1991). From our results, it is now reproducibly possible to initiate callogenesis and somatic embryogenesis within 6 to 8 months of culture on MD3 medium using low plant growth regulator levels. Also, in this condition several somatic embryos have been developed into complete plants when transferred to medium with very low level or without hormones (Fig. 1d). This result supports the efficiency of this new culture medium and can be extended to other palm species.

In conclusion, this report describes the first establishment and maintenance for a long time of regenerable embryogenic culture on MD3 medium containing twenty times lower content of growth regulators than those customarily used in date palm

and other palm species for somatic embryogenesis. Fast embryogenic culture establishment should maintain the genetic integrity and minimize the loss of regeneration capacity (Lührs and Lorz 1988, Gaponenko *et al.* 1988, El Hadrami *et al.* 1991).

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