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Effect of Auxin-Cytokinin Interaction on Micropropagation of Date Palm

Abdul Amir Mater
Department of Biology, College of Science, University of Basrah, Basrah, Iraq

Abstract. In vitro micropropagation of date palm produced single-root-shoot plantlets on media containing activated charcoal. To produce vigorous multiple-root-shoot plantlets, structural modifications have been done by induction of adventitious rooting and axillary shoot growth through the reculture of somatic embryos to media devoid of charcoal but containing various combinations of NAA and BA. Four measurements were conducted through this study, and revealed the presence of significant interactions between auxin and cytokinin.

Average number of adventitious roots per plantlet was 6.2-6.4 at 0.1-1 mg/l NAA and 0 mg/l BA. This optimum range decreased drastically when NAA was changed to higher or lower concentration or BA increased to 1 mg/l. Higher levels of BA suppressed root formation at all levels of NAA combinations.

Average number of shoots per plantlet was 3.0-3.2 at 0-0.01 mg/l NAA and 0 mg/l BA. This optimum range decreased sharply when NAA or BA concentrations were increased.

Optimum growth as expressed by average fresh weight attained per plantlet ranged from 2.650 g at 0.01 mg/l NAA with 0 mg/l BA to 2.812 g at 1 mg/l NAA with 1 mg/l BA. Differences between averages at 0 and 1 mg/l BA changed in magnitude and direction as NAA was increased from 0 to 10 mg/l. Growth was almost inhibited at higher levels of BA with higher levels of NAA.

Average number of proliferating embryos per original plantlet was 13.0-10.4 at 0.01-0.1 mg/l NAA and 0 mg/l BA. This range decreased sharply at the higher levels of both factors.

Introduction

During the last two decades, extensive attempts have been made to propagate date palm by means of tissue culture [1-8]. A substantial success has been reported by the discovery of asexual embryogenesis in callus tissues of date palms [4].

However, the source of callus tissue was the immature seeds and not derived from vegetative parts of the palm. Later on, the most successful vegetative micropropagation was experimented by Tisserat [5]. Along with other investigators, they succeeded to produce date palm plantlets from embryogenic nodular callus tissue originated from offshoots [9]. Histological studies on the development of adventive embryos from the callus proved that callus nodules were the precursors of the vegetative proembryos. They were forced to originate from single meristematic cells located in the epidermal portion of the callus aggregates grown on basal medium suplemented with 100 mg/l 2,4-D, 3 mg/l 2ip and 3 g/l activated char COAL [10].

In 1986, Mater [11] confirmed the results of Tisserat *et al.* [9] concerning plantlet production in vitro. He used quarters of the shoot tips (fragmented shoot tips) isolated from date offshoots to produce the embryogenic callus on a medium containing high level of the auxin 2,4-D (10-100 mg/l); 2 mg/l of each of the cytokinins BA and kinetin and 3 g/l activated charcoal. The plantlets were produced first; by subculturing the embryogenic callus to a medium containing 0.1 mg/l NAA and second; by transfering the mature callus nodules to fresh medium where they germinated and produced whole date palm plants [11]. Young leaf pieces excised from the heart of offshoot without destroying the source material were also used as leaf explants. When cultured on medium containing 100 mg/l 2,4-D with charcoal they usually turned brown and died. However, occasionally few cell layers of callus were formed at the wounded margins. If such callus cells were cultured in agitated liquid medium before browning of the pieces; they initiated cell suspensions from which a large number of somatic embryos were differentiated and subsequently produced plantlets [12].

Tisserat [13] studied the factors involved in the production of plantlets from date palm callus cultures, in order to find a suitable procedure for rapid propagation of free living date plantlets from callus tissue. He observed that plantlet initiation from embryogenic callus was related to auxin pretreatments. He also found that adventitious rooting of the plantlets in vitro could be improved by subculturing the isolated embryos to a medium containing 0-10 mg/l NAA or IAA devoid of charcoal. The optimum adventitious rooting responses and survival in free living conditions were obtained by reculturing the embryos on a medium containing 0.1 mg/l NAA for 8-16 weeks.

Axillary shoot growth were found to be common on a variety of media [13]. In a recent study, plantlets 2-4 months old produced additional shoots in 30-40% of the cultures. Two to three shoots per plantlet were the most common when grown on a medium supplemented with 0.1 mg/l NAA. Attempts to increase the number of shoots with cytokinin were not significant. Histological examination revealed that these additional shoots were derived from lateral bud proliferations [14].

It appeared that the most important factor limiting the in vitro date palm production was the plantlet survival in free living conditions. This was obviously related to vigor of rootshoot system or density of adventitious rooting and axillary shoot growth in plantlets. Since the previous reports provided almost no information concerning the quantitative measurements of this density as influnced by auxins and cytokinins. The present study is a continuation to the previous qualitative study [15]. The objective was to investigate the effects of NAA, BA and their possible interactions on number of roots, number of shoots per somatic embryos and other related parameters during the in vitro micropropagation of the date palm.

Materials and Methods

Production of somatic embryos

Dissected offshoots of the date palm *Phoenix dactylifera* L. cv. Hallawi were used as a source for isolating shoot tips. Segment explants consisting of quarters of the shoot tip were employed for generating embryogenic nodular white callus tissue. The callus was propagated and maintained on a medium containing high level of 2,4-D (10 mg/l) with charcoal in the dark. Initiation of somatic (vegetative) embryos were promoted by subculturing the callus to a medium containing low level of NAA (0.1 mg/l) with charcoal in the light. Uniform embryos averaging 5 mm long were harvested periodically from the cultures [11].

Induction of adventitious roots and axillary shoots

Harvested embryos were recultured individually on a fresh medium containing NAA and charcoal to produce single-root-shoot plantlets, and on media containing various combinations of the auxin NAA with the cytokinin BA without charcoal to produce multiple-root-shoot plantlets. In the latter, NAA was used as one factor at 0, 0.01, 0.1, 1, and 10 mg/l concentration levels in combination with BA as a second factor at the levels of 0, 1, 10 and 100 mg/l; and the procedure was continued according to the previous study of Mater [15].

After three months of incubation in the light, fresh weights of plantlets were recorded, and number of adventitious roots (including the primary root) and developing shoots (including the main shoot) per plantlet were counted in all cultures of the various NAA-BA combinations. In some cultures where original embryos produced callus which in turn produced further proliferating embryos, number of those extra embryos per culture were also counted.

Data collected from this experiment were subjected to factorial analysis of variance at the 5% level of probability. The auxin NAA was considered as the A-factor at 5 levels and the cytokinin BA as the B-factor at 4 levels. The main effect of each factor and their interactions were calculated and expressed in term of F_A, F_B, F_{AB} values respectively, and the simple effects were tested by LSD values according to Steel and Torrie [16, pp. 194-231].

The research was conducted at the Department of Horticulture., College of Agriculture, University of Basrah.

Results

Growth on a media containing activated charcoal

Embryogenic callus tissue was produced after an incubation period of 8-10 months from the beginning of the original culturing of explants on a medium containing 3 g/l activated charcoal and 100 mg/l 3,4-D. This period was found to be essential for the initial callus to be converted to an embryogenic one. Callus aged less than 6 months had no embryonic structures. Propagation of callus was best when cultured on a medium containing 10 mg/l 2,4-D in the dark. Differentiation of somatic embryos from the embryogenic callus on a medium containing 0.1 mg/l NAA in the light produced few embryos (averaging 5 mm long) per culture every one and half months. Reculturing of individual embryos to a fresh medium resulted in elongation of cotyledonary sheath and growth of primary roots (Fig. 1). Entire signle-root-shoot plantlets were produced after 2-3 months where they became ready for transplanting to free living conditions (Fig. 2).

Growth on a media devoid of charcoal, containing NAA-BA combinations

Reculture of somatic embryos (averaging 5 mm long) on a media without charcoal but containing various combinations of NAA and BA resulted in different developmental patterns of growoth. Adventitious root formation and axillary shoot development were obtained in different treatment combinations (Fig. 3).

The effects of 5 levels of NAA combined with 4 levels of BA, on number of roots/somatic embryo were summarized in Table 1. Criterion test of significance revealed that both factors are not independant from each other as indicated by the presence of significant interaction between NAA and BA ($F_{AB} = 2.190^*$). The main effect of the cytokinin BA was also significant ($F_B = 10.260^*$), while the effect of auxin NAA was not significant ($F_A = 2.178$ ns). The significant interaction between NAA and BA indicates that the effect of NAA on number of roots/embryo depended on BA level and vice versa. BA at high concentrations (10 and 100 mg/l) suppressed root formation at all levels of NAA (mean=0.000) while BA at 1 mg/l allowed for very little rooting (mean=0.120 roots/embryo). Meanwhile BA at 0 mg/l gave the highest average number of roots/embryo (mean=3.000). In the absence of BA, average number of roots/embryo depended on NAA levels i.e. 6.4 and 6.2 roots/embryo

Fig. 1. Somatic embryos resulting from germination of callus nodules showing elongation of cotyledons and growth of primary roots.

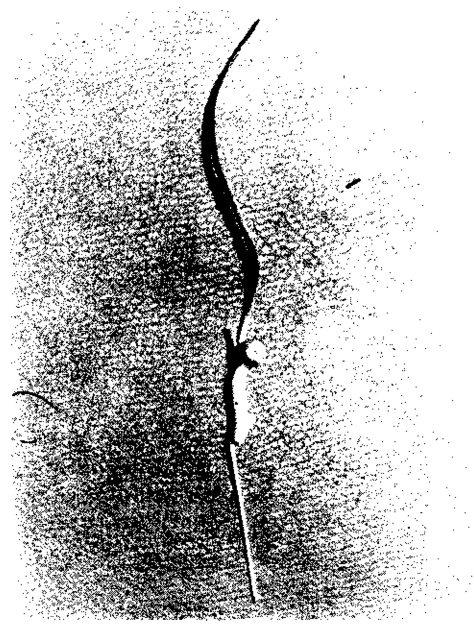

Fig. 2. Single-root-shoot plantlet (3 months old) produced from reculturing individual somatic embryo on a medium containing 0.1 mg/l NAA with charcoal in the light.

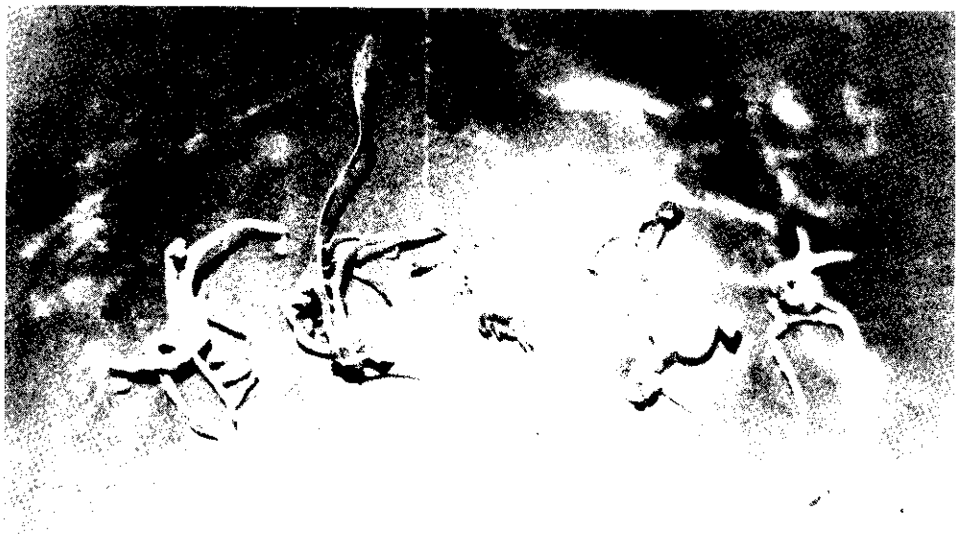

Fig. 3. Multiple-root-shoot plantlets (3-months old) produced from reculturing individual somatic embryos on a media containing 0.1 mg/l NAA and 0·1 mg/l BA without charcoal in the light.

at 1 and 0.1 mg/l NAA respectively. These values were significantly different from these obtained at higher or lower concentrations of NAA as indicated by LSD value. Thus they represent the preferable range for root formation.

The effects of NAA and BA combinations on number of shoots/embryo revealed signficant main effects (F_A=24.061* and F_B = 34.392*) and exerted significant interaction on each other (F_{AB}= 3.936*) (Table 2). BA at the highest concentration (100 mg/l) suppressed shoot growths at all levels of NAA (mean = 0.000 shoots/embryo).

Likewise, NAA at the highest concentration used (10 mg/l) suppressed shoot growth at all levels of BA (mean = 0.000). In general, number of shoots/embryo increased as the level of BA or NAA decreased. The significant interaction between the two factors showed that differences between average numbers of shoots/embryo at 0 and 1 mg/l BA increased in magnitude as NAA levels increased from 0 to 0.1. Also the differences between these averages at low levels of NAA changed in magnitude (increased then decreased) as BA level increased from 0 to 1, and from 1 to 10 mg/l respectively. Differences between average numbers of shoots/embryo (simple effects) at the low levels of the two factors (BA at 0 and 1 mg/l, and NAA at 0 and 0.01 mg/l) were not significant. However, averages of these four combinations were significantly different from most of the averages in the other combinations as indicated by LSD value. Thus they represent the preferable ranges of hormonal balance for shoot growth.

Table 1. Effects of NAA and BA combinations on number of roots per somatic date palm embryo recultured on a media devoid of charcoal.

BA (mg/l)	Number of roots per embryo NAA (mg/l)					mean	F
	0	0.01	0.1	1	10		
0	1.00	1.40	6.20	6.40	0.00	3.00	A=2.178 ns B=10.260*
1	0.20	0.20	0.20	0.00	0.00	0.12	AB=2.190*
10	0.00	0.00	0.00	0.00	0.00	0.00	
100	0.00	0.00	0.00	0.00	0.00	0.00	
mean	0.30	0.40	1.60	1.60	0.00	LSD	2.91

Table 2. Effects of NAA and BA combinations on number of roots per somatic date palm embryo recultured on a media devoid of charcoal.

BA (mg/l)	Number of shoots per embryo NAA (mg/l)					mean	F
	0	0.01	0.1	1	10		
0	3.00	3.20	2.20	0.40	0.00	1.76	A=24.061* B=34.392*
1	2.40	1.60	0.00	0.00	0.00	0.80	AB=3.936*
10	0.20	0.00	2.00	0.00	0.00	0.44	
100	0.00	0.00	0.00	0.00	0.00	0.00	
mean	1.40	1.20	1.05	0.10	0.00	LSD	1.96

The effects of NAA and BA combinations on fresh weight attained per embryo after three months of incubation in the light indicated that the main effects of both factors and their interaction were significant ($F_A = 6.571^*$, $F_B = 13.947^*$, and $F_{AB} = 3.678^*$) (Table 3). The growth of somatic embryos as expressed by fresh weight/embryo was inhibited by treatment combinations of high concentrations of BA (10 and 100 mg/l) with high concentrations of NAA (1 and 10 mg/l) as compared to other combinations of low concentrations of BA (0 and 1 mg/l) with low concentration of NAA (0, 0.01 and 0.1 mg/l). Thus, the later BA/NAA combinations represent the proper ranges of hormonal balance for attaining optimum fresh weight per embryo.

Significant interaction between the two factors at their low levels revealed that the differences between averages of fresh weight/embryo at 0 and 1 mg/l BA were changed in both magnitude and direction as NAA concentration increased from 0 to

Table 3. **Effects of NAA and BA combinations on fresh weight of somatic date palm embryo recultured on a media devoid of charcoal.**

BA (mg/l)	Fresh weight of embryos(g) NAA (mg/l)					mean	F
	0	0.01	0.1	1	10		
0	0.990	2.650	1.036	0.666	0.100	1.088	A=6.571*
1	1.012	1.150	1.350	2.812	0.442	1.353	B=13.947*
10	0.088	0.986	0.438	0.362	0.124	0.400	AB=3.678*
100	0.328	0.320	0.266	0.178	0.078	0.234	
mean	0.604	1.276	0.772	1.004	0.186	LSD	0.905

10 mg/l. With the absence of BA (0 mg/l), average fresh weight/embryo attained its highest value at 0.01 mg/l NAA. This value (2.650g) was significantly different from these values obtained at higher or lower concentrations of NAA. This simple effect of NAA was changed and the highest value occurred at 1 mg/l NAA when BA was increased to 1 mg/l. The latter average of fresh weight/embryo was also significantly different from those at higher or lower concentrations of NAA.

The effects of NAA-BA combinations on the number of proliferating embryos per original somatic embryo showed that the main effect was significant for both factors (F_A = 9.710* and F_B = 24.959*) (Table 4). Number of proliferating embryos/original embryo decreased as BA concentrations increased from 0 to 100. The significant

Table 4. **Effects of NAA and BA combinations on number of proliferating embryos from somatic date palm embryo recultured on a media devoid of charcoal.**

BA (mg/l)	Number of proliferating embryo NAA (mg/l)					mean	F
	0	0.01	0.1	1	10		
0	3.60	13.00	10.40	0.40	0.20	5.52	A=9.180*
1	2.00	1.40	1.80	0.20	0.00	1.08	B=24.959*
10	0.40	1.00	0.20	0.00	0.00	0.32	AB=6.107*
100	0.00	0.00	0.40	0.00	0.20	0.12	
mean	1.50	3.85	3.20	0.15	0.10	LSD	7.156

icant interaction between the two factors(F_{AB} = 6.107*) revealed that diffrences between averages of these extra embryos proliferated at 0 and 1 mg/l BA increased in magnitude to a maximum value as NAA increased from 0 to 0.01 mg/l, and decreased thereafter when NAA was further increased to 10 mg/l. The highest value attained at the combination of 0.01 mg/l NAA and 0 mg/l BA (13.000 embryos/original embryo) was significantly different from those values obtained at lower or higher than 0.01 mg/l NAA. Nevertheless, the differences were insignificant when BA concentration was increased to 1 mg/l or higher.

Discussion

Results of culturing somatic date embryos at an early stage of development (when cotyledonary sheath reached about 5 mm long) on media devoid of charcoal but containing different combinations of NAA with BA showed distinct growth and developmental responses as combinations changed from one treatment to another. Measuring the four observed responses (number of adventitious roots, axillary shoots, proliferating embryos, and fresh weight) at 5 levels of NAA combined with 4 levels of BA revealed the presence of an interaction between NAA and BA at all the four measured parameters.

The auxin NAA proved to be an important plant hormone for induction of adventitious rooting in date palm plantlets [13-15]. The cytokinin BA, which is well known to enhance shoot growth in plants, was not as critical as NAA in its effect on axillary shoot growths in date palm plantlets [13,14].

The significant interaction between NAA and BA on adventitious rooting of embryos indicated a severe suppression of adventitious rooting at high concentrations of BA or NAA. This was expected to occur since high concentrations of auxin or cytokinin or both inhibited vegetative growth and shifted the culture to callus growth [9,11].

However, at low concentrations the highest average number of roots per embryo occurred when BA was at 0 mg/l and NAA kept between 0.1-1 mg/l (Table-1). This result indicates that BA must be excluded if the objective is to attain maximum rooting regardles of shoot growth.

The interaction effect on axillary shoot growth also showed that any of NAA or BA at its highest concentrations used suppressed shoot growth at all levels of the other factor. This complicated relationship restricted normal shoot growth to those combinations at the lower concentrations of NAA and BA (Table 2). Highest average number of shoots per embryo occured when BA concentration was between 0-1 mg/l and NAA between 0-0.01 mg/l. This result indicates that NAA must be lowered to the range 0-0.01 mg/l if the objective is to attain maximum shoot growth within BA range of 0-1 mg/l.

Accordingly, combined optimum of adventitious rooting and axillary shoot growth could be obtained at NAA concentrations between 0,01-1 mg/l and BA excluded from the media. Practically small amounts of NAA or BA are always absorbed and carried by the recultured embryos from the last culture vessels to the fresh media. This may sufficiently account for the interpretation of the uncertained results or the contradictory observations about the BA effects in the previous reports [13-15].

A third significant interaction between NAA and BA levels as measured by embryo fresh weight indicated embryo growth was inhibited by those combinations at high levels of both factors. The optimum fresh weight was reached at BA combinations between 0-1 mg/l and NAA between 0-0.1 mg/l (Table 3). These wide ranges of concentrations for obtaining average fresh weights paralleled the former range of concentrations for obtaining optimum adventitious rooting and axillary shoot growth. Such results ascertain the fact that the highest fresh weight per embryo could be expected from the densely multipled-root-shoot plantlet.

The fourth significant interaction detected between NAA and BA was on the number of extra embryos proliferating from original recultured embryo. With the increased levels of both factors the number was decreased. When BA concentrations were between 0-1 mg/l and NAA between 0-0.1 mg/l, number of proliferating embryos per original embryo reached their highest values (Table 4). Since these accompanied embryos are structurally weaker and nutritionally competing with the original one, they were considered undesirable.

It is obvious that NAA concentrations between 0.01-0.1 mg/l and BA at 0 mg/l which were shown to be the optimum ranges for multiplication of root-shoot growths were also encouraging the proliferation of undesired embryos. The latter was very much minimized when BA concentration was shifted from 0 to 1 mg/l which was shown to be less optimized for root-shoot multiplication in date palm plantlets.

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تأثير تداخلات الأوكسين والسايتوكايتين على الإكثار الدقيق لنخلة التمر

عبدالأمير مهدي مطر
قسم علوم الحياة، كلية العلوم، جامعة البصرة، البصرة، العراق

ملخص البحث. أنتج الإكثار الدقيق لنخلة التمر نباتات مفردة الجذر والساق في بيئات غذائية تحتوي على الفحم المنشط. ولإنتاج نباتات قوية متعددة الجذور والسيقان فقد أجريت تعديلات هيكلية بتحفيز نمو الجذور العرضية والسيقان الإبطية عن طريق إعادة زراعة الأجنة الخضرية في بيئات غذائية تخلو من الفحم وتحتوي على تراكيز مختلفة من NAA و BA.

أظهرت القياسات الأربعة التي أجريت خلال هذه الدراسة وجود تداخلات معنوية بين الأوكسين والسايتوكاينن في التأثير على نمو وتطور الأجنة الجسدية :

١ ـ كان أعلى معدل لعدد الجذور العرضية في النبات الواحد بين ٦,٢ ـ ٦,٤ في التركيزين ١,٠ ـ ١ ملجم/ لتر NAA مع صفر ملجم/ لتر BA. وانخفض هذا المدى بشكل مفاجىء عند تغير مستوى NAA إلى تركيز أعلى أو أدنى أو زيادة BA إلى التركيز ١ ملجم/ لتر. وثبطت المستويات العالية من BA تكون الجذور عند جميع المستويات المؤتلفة من NAA.

٢ ـ كان أعلى معدل لعدد السيقان الإبطية في النبات الواحد بين ٣,٠ ـ ٣,٢ في التركيزين صفر ـ ٠,٠١ ملجم/ لتر NAA مع صفر ملجم/ لتر BA. وانخفض هذا المدى بشكل حاد عند زيادة تركيز NAA أو BA. وثبط المستوى الأعلى لأي عامل منها نمو السيقان عند جميع المستويات المؤتلفة من العامل الآخر.

٣ ـ أما أفضل نمو كان مقدر بدلالة معدل الوزن الرطب المتحقق في النبات الواحد فقد تراوح من ٢,٦٥٠جم في التركيز ٠,٠١ ملجم/ لتر NAA مع صفر ملجم/ لتر BA إلى ٢,٨١٢ جم في التركيز ١

ملجم/ لتر NAA مع ١ ملجم/ لتر BA. وتغيرت الاختلافات بين المعدلات عند التركيزين صفر و ١ ملجم/ لتر BA في القيمة والاتجاه عند زيادة NAA من صفر إلى ١٠ ملجم/ لتر. وتثبط النمو تقريبًا، في المستويات العالية من BA المؤتلفة مع المستويات العالية من NAA.

٤ ـ كان أعلى معدل لعدد الأجنة المتخلفة من النبات الأصلي الواحد (صفة غير مرغوبة) بين ١٣,٠ ـ ١٠,٤ عند التركيزين ٠,٠١ ـ ٠,١ ملجم/ لتر NAA مع صفر ملجم/ لتر BA. وانخفض هذا المدى العالي بشكل حاد عند المستويات العالية لكلا العاملين.