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Investigations on Some Factors affecting the *in vitro* Clonal Propagation of Baladi lime (*Citrus aurantifolia* Swingle).

Tagelsir I.M. Idris[1], Awadia M. Abdelmutalib[1], Elfatih M. Mahdi[2] and Manahil M. Saadalla[3]

[1]Dept. of Horticulture, Sudan University of Science and Technology. [2] Dept. of Horticulture, University of Khartoum. [3] Dept. of Agricultural Sciences, University of Juba.

Abstract

This study examined the impact of some factors on the *in vitro* clonal propagation of Baladi lime. According to results, shoot apices responded better than nodal explants in two growth media containing different concentrations of auxin and cytokinin. In initiation media devoid of auxin, different concentrations of BA and kinetin failed to induce adequate shoot proliferation. Upon transfer of these cultures to a unified auxin-containing multiplication medium, different multiplication rates were obtained based on the type and concentration of the cytokinin in the preceding initiation media with preference to BA at 3 mg/l and kinetin at 3-5 mg/l. Leaf abscission after the fourth week which was attributed to ethylene accumulation in the culture vessels was controlled by supplementing culture media with silver nitrate or sodium meta-bisulfite, but silver nitrate reduced the values of growth parameters.

Key words: *In vitro*, *Citrus aurantifolia*, explant, initiation, cytokinins, ethylene.

Introduction

The tropical climate, soil suitability and water adequacy in Sudan favor the production of most citrus species especially lime and grapefruit. The citrus industry in the Sudan is still at its infancy. Limes (*Citrus aurantifolia* Swingle), originated in India and spread to other tropical and subtropical regions (Salunkhe and Desai, 1984). They are heat tolerant (Sauls, 1998), and can be grown on poor soils provided that good drainage prevents water logging (Sethpackdee, 1992). Extensive lime production is practiced throughout Sudan where they are commonly propagated by seeds and are rarely propagated by vegetative means. As lime's seeds lose viability within few weeks after seed extraction, commercial large scale propagation is normally bound by seasons of fruit availability.

Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plants including citrus. Plant regeneration protocols have been developed for different citrus genotypes from different sources of explants (Gmitter *et al*., 1992). *In vitro* propagation has therefore been a competent tool to overcome problems related to citriculture (Hidaka and Omura, 1989), including mass multiplication (Oliveira *et al.*, (2010), eradication of viral diseases (Crosser and Chandler, 2000) and transgenic plant regeneration (Almeida *et al.*, 2003). However, micro-propagated citrus are difficult to root (Usman, *et al.*, 2005).

The aims of this research were to compare the response of lime shoot explants to the *in vitro* culture, beside the impact of cytokinins in initiation media on cultures prolific capacity in the multiplication phase and to assess

ethylene inhibitors to counteract leaf abscission in culture vessel.

Materials and Methods

Branches were obtained from a mature lime tree of excellent fruiting qualities grown in fruit orchard, College of Agricultural Studies at Shambat. Branches were first washed under tap water for thirty minutes to remove surface dust, thereafter 1.0 cm shoot tips and 1.0 cm nodal segments were excised. Explants were disinfested for 20 minuets in 10% Clorox solution containing two drops of Tween 20 per 100 ml. Explants were rinsed thrice with sterile distilled water to remove the traces of the disinfectant under a laminar air flow hood. Unless otherwise specified media were composed of MS (Murashige and Skoog, 1962) inorganic salts supplemented with 3% sucrose, 7 g/l agar-agar, and in mg/l: Inositol, 100; Thiamine HCl, 1; Pyridoxine HCl, 0.5; Niacin, 0.5; Glycine, 2.0. Growth regulators were used as stated in each test. 50 ml media aliquots were dispensed in Magenta GA7-3 culture vessels and autoclaved for 20 minutes at 121° C. A culture vessel containing 2 explants was considered a replicate, and each treatment was composed of 10 vessels. Cultures were maintained under cool white fluorescent lamps providing 1000 lux at 16 hours day length and 25±1°C constant temperature.

Test of types of explants in two growth media:

Shoot apices were compared against nodal segments in two media. The first medium contained 5 mg/l BA and 0.2 mg/l NAA. The second medium contained 1 mg/l BA and 0.1 mg/l NAA.

BA and kinetin concentrations test in initiation phase:

This test was conducted to find out the benefit of inclusion of cytokinins in initiation culture media. BA and kinetin were tested in the following concentrations: 0, 1, 3, 5 and 7 mg/l.

Response of BA and kinetin treated cultures in initiation phase upon transfer to a unified lime multiplication medium:

This test aimed to study the influence of the type and concentration of cytokinins in initiation culture on the multiplication capacity of lime. Cultures initiated in different concentrations of BA and kinetin were transferred after six weeks to a unified lime multiplication medium containing 1 mg/l BA + 0.5 mg/l kinetin and 0.1mg/l NAA.

Test of ethylene inhibitors:

This test was initiated to counteract the observed leaf abscission of lime after the fourth week in culture which was attributed to ethylene accumulation in the gas phase of culture vessel. In media containing 1 mg/l BA + 0.5 mg/l kinetin and 0.1 mg/l NAA, the following anti-ethylene treatments were tested: 152 mg/l sodium meta-bisulfite; 1 mg/l silver nitrate ($Ag\ NO_3$) and a control.

Design and analysis:

The tests were conducted in factorial or complete randomized design. Wherever appropriate, data were collected for number of shoots, shoot length, number of leaves and number of abscised leaves. Analysis of variance was performed for the completely randomized design and means were separated by Duncan Multiple Range Tests.

Results

As shown in Table 1, shoot tips responded better than nodal segments explants in both media. In medium 2, tips produced more shoots coupled with increase in shoot length. However, tips in medium 1 and nodes in both media shared the second position for shoot length. Shoot tips in medium 1 resulted in the highest number of leaves. Tips and nodes in medium 2 shared the second rank. The lowest number of leaves/culture resulted from nodes in medium 1.

In initiation media, variations in BA concentrations were not accompanied by significant differences in shoot number (Table 2). BA at 3.0 mg/l was slightly better than

other BA concentrations but induced significant increase in shoot number compared to kinetin concentrations except the 1 mg/l kinetin treatment. Although the 5 mg/l kinetin treatment resulted in the longest shoots, it did not differ from the control. All BA treatments increased leaf number significantly compared to the control and kinetin treatments except the 3 mg/l kinetin treatment (Table 2).

Upon transfer to a unified multiplication medium, the type and concentration of cytokinin used in the preceding media affected the morphogenic responses of cultures. The highest number of shoots resulted from the 3 mg/l BA treatment with significant variation from the other treatments. Kinetin at 3 and 5 mg/l ranked second for this parameter without significant difference from the 7 mg/l kinetin treatment. Kinetin concentrations 3-7 mg/l increased shoot length significantly compared to BA and control treatments. However, the 3 mg/l BA treatment ranked top for number of leaves per culture. It is noteworthy to recognize the deteriorative effects of the 5 and 7 mg/l BA treatment compared to the 3 mg/l BA treatment (Table 3).

As shown in Table 4, tip explants responded better than nodes in each of the 3 treatments. The highest number of shoots was obtained from tips in a medium supplemented with sodium meta-bisulfite. Shoot length followed the same trend and was significantly higher for tips and nodes when sodium metabisulfite was added to media. Leaf number was highest when nodes were employed in a sodium-metabisulfite-containing medium. However, leaf abscission was reduced by incorporation of either silver nitrate or sodium meta-bisulfite in culture media (Table 4).

Discussion

In the first test of explants in two growth media, shoot tips performed better than nodal sections in both media. Tissues of tips are young with fast growth ability due to their juvenile nature. Meristematic tissues in the apices are sites of active cell division and immediately underneath are regions where cell determination and differentiation and organs primordia start to appear. This physiological activity necessitates active absorption and assimilation of nutrients. Nevertheless, the results of this study favor the use of lime shoot tips as primary explants and match that of Rana and Singh (2002). On the other hand, the shoot number parameter was enhanced in the second medium which was characterized by low inputs of auxin and cytokinin. This result is in accordance with that of Alkhayri and Al-Bahrany (2001) where 1 mg/l BA was used, fortified by 0.5 mg/l kinetin for lime's proliferation.

In the second and third tests where the influence of various BA and kinetin concentrations were examined, shoot numbers were not affected by either growth regulator or its concentrations in initiation media. This might owe to the slow responses of nodal sections employed in these tests or lack of auxins. It is noteworthy that nodes performed better in the first test where both media contained auxin. Therefore, upon use of nodes as primary explants, direct shoot proliferation should not be expected whatever the type and concentration of cytokinin used unless auxins are incorporated in media. This assumption was further supported by the succeeding subculture test as cultures initiated in media containing various concentrations of BA and kinetin performed better when transferred to a unified auxin-containing lime multiplication medium as suggested by Al-Bahrany (2002). However, the variation in their responses might owe to the type and concentration of cytokinins in the preceding initiation media. The benefits of initiation in 3 mg/l BA compared to kinetin might owe to the efficacy of BA as reported by George *et al.,* (2008). The relative decline in multiplication associated with higher cytokinin doses (5 and 7 mg/l BA; 7 mg/l kinetin) might be explained by beyond optimal inhibitory effects of high cytokinin doses (Alkhayri and Al-Bahrany, 2001).

The ethylene inhibitors test revealed the benefit of the addition of either sodium meta-bisulfite or silver nitrate to prevent leaf

abscission. The leaf abscission had been encountered in all tests especially after the fourth week and was attributed to accumulation of ethylene in the gas phase of lime's culture vessels. Nevertheless, similar beneficial ethylene inhibitory effects of sodium meta-bisulfite and silver nitrate were reported by Idris (2002) who alleviated the ethylene stress caused by poor ventilation in culture vessels by a supplement of either chemical. The negative impact of silver nitrate on shoot multiplication might be due to the concentration used which seems beyond optimal and therefore further optimization tests are needed. Nevertheless, when we consider the response of shoot tips compared to nodes in this anti-ethylene test, shoot tips also proved to be more active and this is a further confirmation to the findings of the first test

References

Al-Bahrany A.M. (2002). Effect of phyto-hormones on *in vitro* shoot multiplication and rooting of lime (*Citrus aurantifolia* Swingle*). Scientia Horticulturae,* 95: 285-295.

Al-khayri J.M. and Al-Bahrany A.M. (2001). *In vitro* micro-propagation of *Citrus aurantifolia* (lime). *Current Science,* 81(9):1242-1246.

Almeida, W.A.B., Mourao Filho, F.A.A., Pino, L.E.Boscariol, L., Rodriguez, A.P.M.. and Mendes, B.M.J. (2003). Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L.Osbeck. *Plant Science*, 164: 203-211.

Crosser J.W. and Chandler J. L. (2000). Somatic hybridization of high yield, cold hardy and disease resistant parents for citrus root stock improvement. *Horticulture Sciences and Biotechnology*, 75:641-644.

Gmitter, F. C., Grosser, J. W. and Moore, G. A. (1992). Citrus .In: Hammerschlag, F.A. and Litz, R.E. (eds.), Biotechnology of Perennial Fruit Crops .Wallingford ,U. K. ;CAB International pp. 335-369.

Hidaka,T. and M.Omura (1989). Control of embryogenesis in citrus cell culture, regeneration protoplast and attempts to callus bank. *Bulletin of the Fruit Tree Research Station Series,* Okitsu, Japan, 16:1-17.

Idris, T.I.M. (2002). *In vitro* culture of pineapple (*Ananas comosus* L). Ph. D. Thesis, Sudan University of Science and Technology.

Oliveira, M.L.P., Febres, V.J., Costa, M.G., Moore, G.A. and Ontoni, W.C. (2009). High efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infilteration. *Plant Cell Reports,* 28: 387-395.

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

Rana, J.S and Singh, R. (2002) . *In vitro* clonal propagation of Kagzi lime (*Citrus aurantifolia* Swingle) through shoot tips. *Progressive Horticulture*, 34:27-34.

Salunkhe , D. K and Desai , B.B ,(1994) Post Harvest Biotechnology of Fruits, Vol. 2. CRC Press Inc., Boca Raton, Florida, USA, 148p

Sauls W. Julian, (1998). Texas Citrus and Subtropical Fruits. Home Fruit Production. *Texas Cooperative Extension,* Dec. 1998.

Sethpakdee, R. (1992). *Citrus aurantifolia*. Christem, Swingle. In: Coranel R.E. and Qverheij, E.W.M. (eds.), Plant Resources of South-East Asia. (Vol. 2): Edible Fruits and Nuts. Prosea foundation, Bogor, Indonesia, pp.126-128.

Usman, M., Muhammed, S. and B. Fatima (2005) *In vitro* multiple shoot induction from nodal

Table 1. *In vitro* growth of lime in two media as affected by the type of explants. Data collected after 6 weeks.

Explant	Media	Shoot number	Shoot length (cm)	Leaf number
Tip	**M1**	4.17 b	1.00 b	5.oo a
	M2	6.00 a	3.50 a	4.oo b
Node	**M1**	2.33 c	1.10 b	2.50 c
	M2	2.33 c	1.22 b	4.17 b

*No significant differences between means with the same letter within column at P=0.05 according to DMRT.

Table 2. The *in vitro* growth of lime as affected by different concentrations of BA and kinetin in initiation media. Data collected after 6 weeks.

Treatment	Shoot No.	New shoot length (cm)	Leaf No.
Control	1.40 abc	1.67 ab	1.90 d
Kin 1 mg/l	1.50 abc	1.02 d	2.60 cd
Kin 3 mg/l	1.00 c	1.23 bcd	3.70 b
Kin 5 mg/l	1.20 bc	2.03 a	2.20 cd
Kin 7 mg/l	1.20 bc	1.53 bc	2.80 c
BA 1 mg/l	1.50 abc	1.46 bcd	4.56 a
BA 3 mg/l	1.96 a	1.00 d	4.90 a
BA 5 mg/l	1.80 ab	1.17 cd	4.80 a
BA 7 mg/l	1.46 abc	0.42 e	4.14 ab

*No significant differences between means with the same letter within column at P=0.05 according to DMRT.

Table 3. *In vitro* growth of lime cultures in a unified multiplication medium as affected by different concentrations of cytokinins in the preceding initiation media. Data collected after 6 weeks.

Cytokinins in initiation media	Shoot no.	New shoot length (cm)	Leaves no.
Control	2.20 d	1.06 c	3.00 de
Kin 1 mg/l	3.20 c	1.40 bc	3.40 cd
Kin 3 mg/l	4.00 b	2.10 a	3.60 cd
Kin 5 mg/l	4.00 b	1.82 ab	4.60 b
Kin 7 mg/l	3.60 bc	1.90 ab	3.60 cd
BA 1 mg/l	4.00 b	1.08 c	4.00 bc
BA 3 mg/l	5.40 a	1.20 c	6.00 a
BA 5 mg/l	2.40 d	0.12 d	2.50 e
BA 7 mg/l	2.00 d	0.14 d	2.60 e

*No significant differences between means with the same letter within column at P=0.05 according to DMRT.

Table 4. *In vitro* growth of lime explants as affected by supplements of Ag NO_3 and sodium meta-bisulfite. Data collected after 6 weeks.

Lime explants	Treatments	Shoot number	Shoot length (cm)	Leaf number	**No of abscised leaves
Tip	**Control**	3.8 b	1.7 c	5.6 b	1.68 a
	$AgNO_3$	2.0 d	2.2 b	5.4 bc	1.20 b
	$Na_2 S_2O_5$	4.8 a	2.7 a	5.6 b	1.00 b
Node	**Control**	2.8 c	1.8 c	4.8 c	1.40 ab
	$AgNO_3$	1.0 e	1.0 d	1.8 d	1.00 b
	$Na_2 S_2O_5$	2.2 d	2.7 a	7.6 a	1.10 b

*No significant differences between means within column with the same letter at P=0.05 according to DMRT.

**Arcsine transformed data by: square root of (X+1).

استقصاء بعض العوامل المؤثرة في التكاثر السلالي لليمون البلدي

تاج السر إبراهيم محمد إدريس[1] عوضية مختار عبدالمطلب[1] الفاتح محمد مهدي[2] ، مناهل مصطفى سعدالله[3]

١. قسم البساتين – جامعة السودان للعلوم والتكنولوجيا
٢. قسم البساتين – جامعة الخرطوم
٣. قسم البساتين – جامعة بحري

أختبرت هذه الدراسة أثر بعض العوامل على الاكثار السلالى لنبات الليمون البلدى فى القوارير. حسب النتائج كانت استجابة قمم السوق أفضل من العقد الساقية كأجزاء نباتية مستخدمة لانشاء الزراعات فى وسطين مختلفين يحتويان تركيزات مختلفة من الأوكسين والسايتوكاينين. فى الأوساط الانشائية الخالية من الأوكسين فشلت التركيزات المختلفة من البنزايل أدنين أو الكاينتين فى اكثار كافى للسيقان، ولكن عند النقل الى بيئة اكثار موحدة تحتوى الأوكسين تم الحصول على اكثار بدرجات متفاوته تأثرا بنوع وتركيز السايتوكاينين فى بيئة الانشاء وكانت الأفضلية للبنزايل أدنين (BA) بتركيز 3 مجم/لتر والكاينتين بتركيز 3– 5 مجم/لتر. أمكن التحكم فى تساقط الأوراق بعد الأسبوع الرابع الذى عزى لتراكم الايثلين فى أوعية الزراعة باضافات من نترات الفضة أو الصوديوم ميتابايسلفايت الى البيئات الا أن نترات الفضة قد خفضت قيم النمو.