Effect of plant growth regulators and malt extract on somatic embryogenesis and in vitro grafting of Citrus plants

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Abstract

Citrus fruits are among the most consumed fruits in the world. This is mainly due to their high content in vitamin C and other health-promoting bioactive compounds. Developing new and efficient propagation systems for Citrus plants is of high importance to rejuvenate old orchards and to satisfy the high demand of consumers. Herein, we aimed to establish an efficient regeneration method through micrografting for Citrus plants. Nucellar embryos of sour orange (*Citrus aurantium* L.) were taken from mature seeds and cultured on plant growth regulator (PGR)-free Murashige and Skoog medium with some modifications (MSm) to be used as rootstock. For scion preparation, undeveloped ovules of sweet orange *Citrus sinensis* (L.) Osbeck cv. Washington Navel were cultured either on PGR-free MSm medium, MSm medium supplemented with malt extract at different concentrations, or on MSm medium supplemented with different PGRs to induce somatic embryogenesis. The findings showed that after 10 days of culture, all nucellar embryos of sour orange were developed into seedlings. Besides, it was found that the number and size of embryos vary in each seed. The number of embryos ranged from 1 to 8, with a degree of polyembryony ranging from 3.08 to 4.12. Regarding sweet orange cv. Washington Navel, the highest percentage of embryogenic callus formation (50%) was observed on the medium containing 500 mg/L malt extract, with no significant difference with media containing 1000 and 1500 mg/L malt extract (46%). However, callus and proembryo morphology and proliferation varied depending on malt extract concentration. Surprisingly, none of the media containing PGRs showed embryogenic callus formation. Micrografting was performed by inserting somatic embryos of sweet orange cv. Washington Navel on decapitated sour orange seedlings. The micrografted plants were transferred to MSm medium supplemented with 1 mg/L gibberellic acid where a survival rate of 100% as well as normal growth and development of plants were observed.

Keywords: *Citrus plants, in vitro, micrografting, nucellar embryogenesis, regeneration, somatic embryogenesis.*

Effets des substances de croissance et de l'extrait de malt sur l'embryogenèse somatique et le microgreffage des agrumes

Résumé

Les agrumes font partie des fruits les plus consommés dans le monde. Ceci est dû à leur teneur élevée en vitamine C et d'autres composés bioactifs bénéfiques pour la santé. Le développement de systèmes de propagation efficaces pour les plantes d'agrumes constitue aujourd'hui un outil fondamental pour rajeunir les vieilles plantations et satisfaire la demande croissante des consommateurs. Le présent travail vise donc à développer un schéma de multiplication in vitro par microgreffage entre le porte-greffe usuel des agrumes, le bigaradier (*Citrus aurantium* L.) et la variété d'agrume commerciale asperme Washington Navel (*Citrus sinensis* (L.) Osbeck). Des embryons nucellaires ont été prélevés de graines matures du bigaradier et cultivés sur le milieu de Murashige and Skoog modifié (MSm) et dépourvu de substances de croissance. Les greffons correspondaient à des embryons somatiques obtenus à partir de culture d'ovules avortés de la variété Washington Navel sur le milieu MSm avec ou sans substances de croissance. Dans ce dernier cas, différentes concentrations d'extrait de malt ont été additionnées. Les résultats ont montré que, après 10 jours de culture, la totalité des embryons nucellaires du bigaradier a germé. En outre, les graines du bigaradier ont montré des différences concernant le nombre et la taille des embryons nucellaires. Le nombre d'embryons par graine a varié de 1 à 8 alors que le degré de polyembryonie était de 3.08 à 4.12. Parallèlement, le taux d'induction des cals embryogènes à partir d'ovules avortés de la variété Washington Navel a varié en fonction du milieu de culture, avec le taux le plus élevé (50%) observé sur le milieu contenant 500 mg/L d'extrait de malt, sans différence significative avec les milieux contenant 1000 et 1500 mg/L (46%), alors que les milieux additionnés de substances de croissance n'ont pas induit de callogenèse. Le microgreffage entre les porte-greffes issus du bigaradier et les embryons somatiques de la variété Washington Navel a montré un taux de réussite de 100% sur le milieu MSm additionnée de 1 mg/L d'acide gibbérellique, avec un développement normal des plantes microgréffées.

Mots-clés : *Citrus, embryogenèse nucellaire, embryogenèse somatique, in vitro, microgreffage, régénération.*

تأثير هرمونات النمو وخلاصة الشعير على تكوين الأجنة الجسدية والتطعيم الأنبوبي للحوامض

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ملخص

تعتبر الحوامض من أكثر الفواكه استهلاكا في العالم وذلك لاحتوائها على نسبة عالية من فيتامين سي ومركبات حيوية أخرى مفيدة للصحة. في هذا الإطار، يعتبر تطوير تقنيات إكثار جديدة وفعالة لنباتات الحوامض ذو أهمية كبيرة لتجديد الأشجار وتلبية الطلب المتزايد من المستهلكين. يهدف هذا البحث إذا إلى تطوير نموذج للتكاثر الدقيق عن طريق تقنية التطعيم الجزيئي بالمختبر بين حامل طعم النارنج (أو البرتقال المر) (*Citrus aurantium* L.) المعتاد استعماله للحوامض، وصنف الحوامض التجاري الخالي من البذور (*Citrus sinensis* Osbeck (L.)) 'واشنطن نافيل'. في هذا الإطار، قمنا بأخذ الأجنة النووية من بذور النارنج الناضجة وزرعها على وسط النمو موراشيغ وسكوج معدل (MSm) والخالي من هرمونات النمو. فيما يخص الطعوم، قمنا باستعمال الأجنة الجسدية المنتجة بعد زراعة بويضات غير مخصبة لصنف 'واشنطن نافيل' على وسط MSm مع أو بدون هرمونات النمو. في حالة عدم استعمال هرمونات النمو، تم إضافة تركيزات مختلفة من مستخلص الشعير. أوضحت نتائج هذه التجارب أنه بعد 10 أيام من الاستنبات، جميع أجنة النارنج نبتت بنجاح. إضافة إلى ذلك، أظهرت بذور النارنج اختلافات في عدد وحجم الأجنة النووية، حيث إن عدد الأجنة تراوح ما بين 1 إلى 8 أجنة لكل بذرة بينما تراوحت درجة تعدد الأجنة من 3.08 إلى 4.12. فيما يخص إنتاج الأجنة الجسدية لصنف 'واشنطن نافيل'، أظهرت النتائج اختلاف معدل إنتاج الأنسجة الجينية من البويضات الغير المخصبة حسب وسط النمو المستعمل، حيث لوحظ أعلى معدل (50%) على الوسط الذي يحتوي على 500 ملغ/لترمن مستخلص الشعير، بدون فرق كبير مع الوسائط التي تحتوي على 1000 و1500 ملغ/لتر (46٪)، في حين أن الوسائط التي تحتوي على هرمونات النمو لم تدعم إنتاج الأنسجة الجينية. بعد القيام بعملية التطعيم داخل المختبر بين النارنج و 'واشنطن نافيل'، تم تسجيل معدل نجاح مرتفع (100%) على وسط النمو MSm المزود ب 1 ملغ/لتر من حمض الجبريليك، كما أظهرت النباتات المطعمة نموا طبيعي.

الكلمات المفتاحية: التطعيم الجزيئي بالمختبر، تكوين الأجنة النووية، تكوين الأجنة الجسدية، إنبات أنبوبي.

Introduction

Citrus fruits are the most produced fruits worldwide, with a production exceeding 135 million tons, and are the main winter fruits consumed in the Mediterranean countries (FAOSTAT, 2017; Rahman et al., 2019). The area of cultivation of Citrus plants is located between 40° North latitude and 40° South latitude. Therefore, Citrus plants have a great capacity to adapt to different soil and climatic conditions (Ollitrault and de Rocca Serra, 1992).

Citrus is one of the most economically important fruit crops in Morocco. Indeed, Citrus orchards cover around 125 000 ha, with an annual production of around 2 million tons (MAPMDREF, 2017). Besides, the Citrus sector generates 25 million working days annually (MAPMDREF, 2017). Unfortunately, the old age of Citrus plantations is one of the problems that have to be resolved (MAPMDREF, 2018). Thus, it is important to renew the old Citrus plantations and to establish new orchards by using rootstock/cultivar associations that are adapted to the soil and climatic conditions of the different Citrus production regions of Morocco, and that will produce fruits of high quality able to meet the international market requirements.

Generally, Citrus rootstocks are produced through seed germination, but scarcely by cuttings or layering. Citrus rootstocks are a key component of the sustainable Citrus cultivation and thus grafting is the most used method for vegetative propagation of Citrus plants. However, it worth noting that many Citrus species that are used for rootstock production have a limited quantity of seeds, which may hamper the multiplication of this plant genus (Bouzid, 1983). On the other, multiplication through stem cutting is difficult due to the recalcitrant nature of the majority of Citrus plants to this technique.

For these reasons, in vitro culture techniques could be used for Citrus propagation since they will allow to overcome the above-mentioned difficulties and thus ensuring massive production of Citrus plants (Bowman and Joubert, 2020; Mahmoud et al., 2020). In fact, such techniques have been widely used for rapid and large-scale propagation of many plant species and thus to rehabilitate groves and ecosystems (Mazri et al., 2019 a,b). In addition, the use of in vitro multiplication techniques allows the production of healthy plants in a relatively short time. In vitro micrografting is one of the in vitro culture techniques that was recommended for Citrus plant production (Murashige et al., 1972). It is the technique by which a scion is grafted onto the rootstock. The choice of the scion must be carried out beforehand according to the agronomic characteristics sought such as compatibility with the rootstock, the speed of fruit setting and the quality of the fruits. On the other hand, the main characteristics looked for in rootstocks are the adaptability to different soils, resistance to biotic and abiotic stresses and the good root system.

Plant growth regulators (PGRs) play a major role in in vitro plant propagation. Indeed, PGRs have been widely used in many plant species for micropropagation through different techniques. For example, PGRs were used for olive somatic embryogenesis (Mazri et al., 2013), date palm organogenesis (Mazri et al., 2019a), and argan micrografting (Koufan et al., 2020). In fact, PGRs are involved in cell division, differentiation and growth (Gaspar et al., 1996, 2003; Feher et al., 2003; Gaj, 2004). On the other hand, malt extract has been recommended for in vitro culture of Citrus

plants. Malt extract is a carbohydrate source that has been used for callus induction, somatic embryogenesis development and plant regeneration in many Citrus species (Sawy et al., 2005; Gholami et al., 2013; Amin and Shekafandeh, 2015).

In the present investigation, we aimed to develop an efficient propagation system for Citrus plants by using the micrografting technique. Thus, we used the sour orange (*Citrus aurantium* L.) as a rootstock source on which somatic embryos of *Citrus sinensis* (L.) Osbeck cv. Washington Navel, induced in vitro, were grafted. Sour orange was chosen due to its tolerance to water deficit, high adaptability to wide-ranging soil conditions, beneficial effect on fruit quality and tolerance to many diseases such as gummosis and exocortis (Emmanouilidou and Kyriacou, 2017; Robles et al., 2017; Raddatz-Mota et al., 2019). During our experiments, the effect of different PGRs, namely 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and 6-(dimethylallylamino) purine (2iP), as well as that of various malt extract concentrations (500, 1000 and 1500 mg/L) on somatic embryogenesis induction from undeveloped ovules of *Citrus sinensis* (L.) Osbeck cv. Washington Navel were evaluated.

Materials and Methods

Culture medium and conditions

The basal formulation used in the present study was that of Murashige and Skoog (1962) culture medium with some modifications (MSm) as follows: Murashige and Skoog (1962) macro-elements, Heller (1953) microelements, and Murashige and Turcker (1969) vitamins. All culture media were supplemented with 20 g/L sucrose and solidified with 8 g/L bactoagar.

The pH of all media was adjusted to 5.6. The cultures were kept under a 16h photoperiod under a 25°C and transferred to fresh medium at 4-week intervals.

Plant material and disinfection

Mature fruits were collected from a sour orange tree (*Citrus aurantium* L.) tree located in the National school of agriculture of Meknes (ENAM, Morocco, 33°50'39.9"N 5°28'38.7"W). Seeds of sour orange were thoroughly washed with tap water then immersed in a mercury chloride solution (1 g/L) for 15 min, followed by three rinses in sterile distilled water. The nucellar embryos were then removed from seeds by using a binocular lens.

As for *Citrus sinensis* (L.) Osbeck cv. Washington Navel, mature fruits were purchased from local markets. The fruits were surface sterilized by immersion in commercial bleach (10%) for 15 min and then sprayed with 95° ethanol and flamed for a few seconds to sterilize the outside part. Thereafter, the fruits were peeled and sectioned longitudinally into several parts. Finally, the undeveloped ovules were extracted under a binocular lens and placed in culture.

Experiments

For rootstock preparation, nucellar embryos of sour orange were cultured on PGR-free MSm medium for one month. We used a total of 25 seeds, and the experiment was repeated twice.

For scion preparation, the undeveloped ovules of *Citrus sinensis* (L.) Osbeck cv. Washington Navel were cultured either on PGR-free MSm medium supplemented with malt extract at different concentrations: 500, 1000 and 1500 mg/L, or on MSm medium supplemented with different PGR combinations: 0.01 mg/L 2,4-D; 0.01 mg/L 2,4-D + 0.1 mg/L BAP; 0.01 mg/L 2,4-D + 0.1 mg/L 2iP or 2 mg/L 2,4-D. Herein, we used 10 petri dishes per treatment, each petri dish contained 5 explants. A malt extract- and PGR-free medium was used as a control.

Somatic embryos obtained from undeveloped ovule-derived calli of *Citrus sinensis* (L.) cv. Washington Navel were grafted in vitro onto the seedlings of sour orange. In vitro grafting was performed as follows:

- The seedlings of sour orange were decapitated and the leaves and axillary buds were removed.
- Under a binocular lens, a 'T' incision was made on the upper half of the rootstock without damaging its vascular system.
- An individual somatic embryo of *Citrus sinensis* (L.) cv. Washington Navel was carefully placed into the incision.
- The micrografted plant was cultured on MSm medium with or without 1 mg/L gibberellic acid (GA_3). In each culture medium, the total number of micrografted plants was 15.

Data collection and statistical analyses

Data were recorded for the degree of polyembryony of seeds of sour orange (calculated as the total number of nucellar embryos divided by the total number of seeds), the percentage of embryogenic callus formation in *Citrus sinensis* (L.) cv. Washington Navel and the percentage of successfully micrografted plants that showed normal growth and development.

All experiments were conducted in a Completely Randomized Design (CRD). Data were analyzed using ANOVA and means were separated using the Student-Newman-Keuls test at the 5% significant level. All data were analyzed by SPSS for windows. Percentage data were arcsine transformed before analysis.

Results and discussion

Root stock preparation

In vitro seed germination

In the present study, seeds of sour orange (*Citrus aurantium* L.) were used as source of rootstock. Sour orange was chosen since it ensures high agronomic performances such as well adaptation to different types of soils, high fruit quality, high productivity of the grafted plants as well as resistance/tolerance to many biotic and abiotic factors (Emmanouilidou and Kyriacou, 2017; Robles et al., 2017; Raddatz-Mota et al., 2019). The germination of nucellar embryos occurred after six days of culture. In fact, we observed cotyledon greening and radicle elongation. After ten days of culture, all embryos were developed into seedlings. The technique used in the present study, by which nucellar embryos are first extracted then placed on culture medium to grow into seedlings showed more efficient results than conventional seed germination. In fact, Sykes (2011) reported an in vitro seed germination percentage of *Citrus aurantium* L. of 40% in genotype Daidai and 45% in genotype Goutoucheng, with the emergence of 1.13 and 1.44 seedlings per seed, respectively. On the other hand, our findings showed that the growth of nucellar embryos depends on their size. In fact, the largest embryos showed faster growth than the smallest ones (Fig. 1). Similar remark was also observed under natural conditions, in which the development of the largest nucellar embryos is often faster than that of the other ones (Frost and Soost, 1968).

Figure 1: Seedlings derived from nucellar embryos of *Citrus aurantium* L. after one month of culture on PGR-free MSm medium under 16h photoperiod

Degree of polyembryony of sour orange

Similarly to the majority of Citrus plants, the seeds of sour orange are characterized by the presence of one or many nucellar embryos. Thus, the number, size and morphology of embryos vary in each seed. Besides, not all the embryos of the same seed develop into a viable plant under natural conditions, but only the largest and most vigorous ones are developed into young seedlings. Generally, the small embryos are unable to develop. This is probably due to the lack of reserves and because of the competition among the different embryos (Frost and Soost, 1968). The use of in vitro culture techniques allows us to determine more precisely the degree of polyembryony of sour orange seeds.

Table 1 showed that the number of nucellar embryos in a mature seed ranges from 1 to 8, with a degree of polyembryony of 3.08 and 4.12 in the first and second repetition, respectively. According to Soares et al. (1993), the degree of polyembryony of a Citrus plant varies depending on many factors including the environment, the species, the cultivar, the fruits of the same plant and the seeds of the same fruit.

Table 1: Number of nucellar embryos and degree of polyembryony in seeds of *Citrus aurantium* L.

	Number of nucellar embryos per seed	Number of seeds	Total number of nucellar embryos	Degree of polyembryony
1st repetition	1	2	2	3.08
	2	6	12	
	3	7	21	
	4	8	32	
	5	2	10	
Total		25	77	
2nd repetition	1	2	2	4.12
	2	2	4	
	3	8	24	
	4	3	12	
	5	2	10	
	6	6	36	
	7	1	7	
	8	1	8	
Total		25	103	

Morphological description of seedlings

Nucellar embryos exhibit a growth vigor correlated with their size. In fact, large embryos produce more vigorous seedlings than those obtained with small embryos. The vigorous seedlings of sour orange have a large internode distance and their leaves are sessile and opposite. Besides, they are characterized by a good root system (i.e. high root volume and deep roots; Fig. 1).

Scion preparation

In this experiment, we evaluated the effects of different malt extract concentrations and PGRs on somatic embryogenesis induction. After 3 months of culture, embryogenic calli (i.e. calli with globular proembryos) were observed. Globular proembryos were linked together by a mucilaginous substance. The highest percentage of embryogenic callus formation (50%) was observed on PGR-free MSm medium supplemented with 500 mg/L malt extract. This was followed by media containing 1000 and 1500 mg/L malt extract, which showed a callus formation rate of 46% (Table 2). On the other, none of the culture media supplemented with PGRs showed embryogenic callus formation. Statistical analysis showed no significant difference in terms of embryogenic callus formation between the media containing malt extract (Table 2).

Malt extract is a carbohydrate source that has shown promotive impact on somatic embryogenesis in Citrus plants (Badr-Elden, 2017). Malt extract was also reported to stimulate the germination of early cotyledonary stage embryos obtained from the in vitro rescue of *Citrus aurantium* (Carimi et al., 1998). According to Badr-Elden (2017), the beneficial effect of malt extract on callus induction is observed only at high concentrations. In fact, the use of a 400 mg/L malt extract resulted in a high regeneration percentage (97.50 %) of callus derived from juice vesicles and the maximum callus fresh weight with 4.78 g/explant. Sawy et al. (2005) recommended the use of malt extract at the concentration of 500 mg/L for callogenesis and somatic embryogenesis from ovules of Citrus. Amin and Shekafandeh (2015) suggested the use of malt extract at concentrations ranging from 500 to 700 mg/L to induce somatic embryogenesis in Mexican lime. Gholami et al. (2013) recommended the combination malt extracts and GA_3 to promote somatic embryogenesis in Citrus plants. According to Hussain et al. (2016), the highest callogenesis rate in kinnow mandarin (90%) was obtained when the culture medium contained both 5 mg/l 2,4-D and 500 mg/l malt extract. On the other hand, Mahadi et al. (2016) suggested the use of the combination of 2 mg/l, 2,4-D, and 2 mg/l BAP to produce embryogenic calli in *Citrus microcarpa*. Our findings showed that combining 2,4-D and BAP did not induce callogenesis. This might be due to the different concentrations of these PGRs used or to the species effect. In fact, it is well known that morphogenesis and regeneration in vitro depend strongly on the species, the genotype, the explant and PGRs. Indeed, exogenous PGRs interact with the endogenous plant hormones and modify their concentrations, which results in cell division, differentiation, growth and morphogenesis. Besides, the endogenous concentration of plant hormones varies among species, genotypes and explants (Gaspar et al., 1996, 2003; Feher et al., 2003; Gaj, 2004).

Table 2: Effect of different culture media on embryogenic callus formation, callus morphology and proembryo proliferation. Data of embryogenic callus formation are means ± standard deviation. Data in the same column followed by the same letter are not significantly different at the 5% significance level

Culture medium	Embryogenic callus formation (%)	Callus and proembryos description
MSm	0 b	- No callus formation
MSm + 500 mg/L malt extract	50.0 ± 21.6 a	- Low amount of white, friable and hydrated callus. - High proliferation of proembryos.
MSm + 1000 mg/L malt extract	46.0 ± 13.4 a	- High amount of yellow, friable and hydrated callus. - low proliferation of proembryos.
MSm + 1500 mg/L malt extract	46.0 ± 16.4 a	- Low amount of yellow, friable and hydrated callus. - Predominance of large proembryos
MSm + 0.01 mg/L 2,4-D	0 b	- No callus formation
MSm + 0.01 mg/L 2,4-D + 0.1 mg/L BAP	0 b	- No callus formation
MSm + 0.01 mg/L 2,4-D + 0.1 mg/L 2iP	0 b	- No callus formation
MSm + 2 mg/L 2,4-D	0 b	- No callus formation

Morphological description of embryogenic calli

Despite that there was no significant difference in terms of the percentage of embryogenic callus formation in media containing malt extract, our observations showed that callus morphology and development vary among these three culture media. Indeed, in the medium containing 500 mg/L malt extract, there was a low amount of friable and hydrated calli (Fig. 2a) but proembryo proliferation was higher than in the other media (containing 1000 and 1500 mg/L malt extract). In the medium containing 1000 mg/L malt extract, the amount of callus was important. Calli were yellow, friable and hydrated but with a low proliferation of proembryos (Fig. 2b). In the medium containing 1500 mg/L malt extract, there was a very low amount of yellow, friable and hydrated callus and the predominance of large proembryos (Fig. 2c).

Although there were differences in terms of callus and proembryo morphology and proliferation depending on malt extract concentration, the proembryos obtained on the three different media were successfully developed into cotyledonary somatic embryos (Fig. 3).

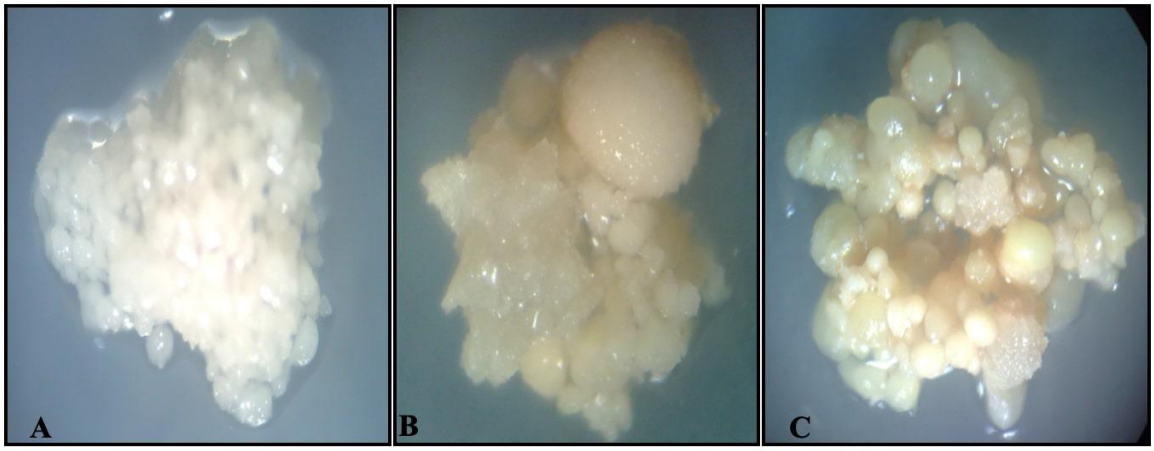

Figure 2: Morphology of callus obtained from *Citrus sinensis* (L.) Osbeck cv. Washington Navel on different culture media. **A**. MSm medium containing 500 mg/L malt extract. **B**. MSm medium containing 1000 mg/L malt extract. **C**. MSm medium containing 1500 mg/L malt extract

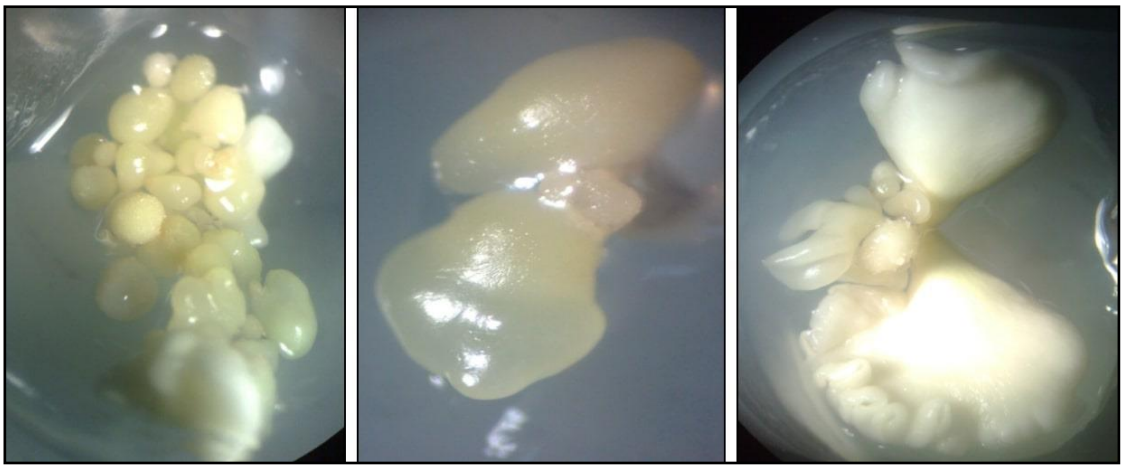

Figure 3: Somatic embryo development on MSm medium containing 500 mg/L malt extract

Micrografting and plantlet development

After one month of culture, all the micrografted plants (100%) cultured on MSm medium supplemented with 1 mg/L GA_3 survived and showed normal growth and development (Fig. 4). In fact, new shoots were produced while no necrosis or contamination were observed. The successful micrografting may be due a high compatibility between the rootstock and scion. Besides, adding GA_3 to culture medium may also play a major role in successful growth and development of micrografted plants. Indeed, in GA_3-free MSm medium, only 13.3% micrografted plants survived. Furthermore, they showed a slow growth. The beneficial effect of GA_3 on plant micrografting was observed in other plant species (Córdova-Risco et al., 2017; Koufan et al., 2020).

Figure 4: In vitro grafting and successful growth and development of the micrografted plants

Conclusions

We reported an efficient in vitro grafting protocol for *Citrus sinensis* (L.) Osbeck cv. Washington Navel, by using sour orange (*Citrus aurantium* L.) as rootstock. Rootstock preparation was carried out on PGR-free MSm medium while scion preparation was achieved on PGR-free MSm medium supplemented with malt extract. Micrografting and subsequent growth and development were successfully achieved with a high survival rate of 100%, which reflects the high compatibility between the rootstock and scion and the appropriate culture conditions. The reported protocol can be used for rapid and efficient propagation through micrografting of the high economically important sweet orange *Citrus sinensis* (L.) Osbeck cv. Washington Navel.

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