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Factors Affecting the Incidence of Vitrification of *in vitro* Propagated Fruit Trees

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Abstract. *In vitro* Clonal propagation of fruit trees to produce large number of plants, often leads with the problem of vitrification or hyperhydric transformation. This physiological disorder was observed in pear, quince, grape and date palm.

The frequency of vitreous shoots was increased in the presence of high BAP and NH_4^+ concentrations, as well as on Murashige and Skoog mineral salts in culture medium. In pear and quince, the formation of normal shoots from vitreous explants was obtained by using mineral salts of Quoirin & Lepoivre supplemented with low concentration of BAP and high concentration of sucrose in the media. In date palm, the traits to correct the vitreous plants were not succeeded, however, the formation of high percentage of vitrification was reduced by using Beauchesne mineral salts in the medium.

A histological study showed in pear that vitreous explants containing a low level of lignification and a limited number of xylem layers in comparison with normal shoots. The role of different factors studied was discussed.

Key words: Vitrification, Tissue Culture, Fruit Trees, Lignification.

Abbreviations: BAP = Benzyl aminopurine, NAA = Naphthallene acetic acid, GA_3 = Gibberellic acid, IBA = Indole butyric acid, PG = Phloroglucinol, PZ = Phlorodzin and MS = Murashige and Skoog.

Introduction

Vitrification or hyperhydric transformation is one of the problems of *in vitro* propagation of fruit trees. It is a physiological disorder frequently affecting *in vitro* propagated plants.

The symptoms during the process of vitrification could be described as follows: The leaves of glassy plantlets become humid, thick and translucent, wrinkled and curled, and easily breakable. Translucency and malformation are due to chlorophyll deficiency and general cell hyperhydricity [1; 2]. Vitrified leaves do not have palisade tissue, but only spongy mesophyll [3]. The whole explants become very turegescent and loose the ability

to grow and initiate root in culture. The vitrified plantlets do not survive after transferring to soil even when kept under mist.

This disorder has been encountered with several fruit trees species such as: Prunus and Malus [4; 5, pp. 126-127]; *Prunus avium* [6]; *Pyrus communis* [7]; *Cydonia oblonga* [8]; *Prunus amygdalus* [9] and *Vitis vinifera* [10, p. 399; 11].

Several physical or chemical signals refer to *in vitro* conditions such as, hormones, gas, ions, water potential, temperature etc., which initiate the vitrification process [2]. Vitreous plant production *in vitro* cannot be commercialized, that explain why most tissue culturist focused their effort on practical means of avoiding vitrification [12].

In this study, several factors influencing the vitrification in some fruit trees such as pear, grape, quince and date palm have been investigated. The different hypothesis which might be put to overcome this phenomenon were reviewed. At the same time, a histological study concerning the role of sucrose in this phenomenon in pear was presented.

Material and Methods

The plant used in these experiments were pear (*Pyrus communis*) cvs "williams" and "passe crassane" quince (*Cydonia oblonga*) cv "provence quince", grape (*Vitis vinifera*) cv "khalas" and date palm (*Phoenix dactylifera* L.) cvs "Hillaly" and "Khalas." The mother plants of pear and quince were placed in a greenhouse under the following conditions: 22°C day and 12°C night temperatures with natural illumination. Regrowth from these plants was used as a source of explants for studies of micropropagation *in vitro*. All detailed of micropropagation process were extensively described previously by Al-Maarri, *et al.* [7; 8].

The mother plants of grape were taken directly from the field and cultured in *in vitro*. The propagation procedures were described previously by Al-Maarri and Al-Ghamdi [11]. Date palm offshoots cv. "Hillaly" and "Khalas" cultivars were taken off from the Date Palm Research Center field in King Faisal University and were used as a source of plant material.

Some factors affecting the phenomenon of vitrification were studied during the propagation of these fruit trees *in vitro* as follows:

Effect of BAP concentrations

This experiment was conducted on pear and quince. The influence of BAP concentrations (0, 0.5, 1, 2 and 4 mg/l) on shoot multiplication and on the quality of shoots formation were studied. Twenty-four explants (15-20 mm) used for each treatment, were inoculated into test tubes (200 × 24 mm) with 15 ml culture medium (A) containing the mineral salts of Quoirin and Lepoivre [13] with the addition (mg/l) of 0.5 IBA; 0.2 GA_3; 0.4 thiamine and 100 inositol, 3% sucrose and solidified with 0.6% agar plus different concentration of BAP.

Effect of NH_4^+ concentrations

This experiment was conducted on grape cv "Khalas". Two treatments were used: first by using medium containing mineral salt of Murashige and Skoog [14] with the addition (mg/l) of 0.5 BAP, 0.2 GA_3, 0.1 IBA, 0.4 thiamine, 100 Inositol, 3% sucrose and 0.6% agar; second by using the same medium but NH_4NO_3 of MS was diluted 4 times.

Effect of mineral salts solution

This experiment was made on pear and date palm. In the case of pear MS [15] and Quorin and Lepoivre [13] mineral salts were used. In date palm MS and Beauchesne [15], salt solution were used. The culture media were supplemented with different combination of vitamins and hormones. All media detailed was described in Table 1.

Table 1. Composition of media used in date palm multiplication

Media composition	Initial culture Media (A)	Bud initiation Media (B)	Bud multiplication Media (C)	Rooting Media (D)
MS salt	MS	MS	MS	MS/3
Sucrose ($g.l^{-1}$)	30	30	30	70
Agar ($g.l^{-1}$)	7	7	7	7
Thiamine HCl ($mg.l^{-1}$)	1	1	1	0.4
Inositol ($mg.l^{-1}$)	100	100	100	100
Pyridoxine ($mg.l^{-1}$)	1	1	1	-
Biotin ($mg.l^{-1}$)	1	1	1	-
Nicotinic acid ($mg.l^{-1}$)	1	1	1	-
Glutamine HCl ($mg.l^{-1}$)	200	200	200	-
Ca Pantothenate ($mg.l^{-1}$)	1	1	1	-
Adenine sulfate ($mg\ l^{-1}$)	45	45	45	-
Kinetin ($mg.l^{-1}$)	-	2	0.2	-
BAP ($mg.l^{-1}$)	-	1	0.1	-
2ip ($mg.l^{-1}$)	0.5	1	0.1	
NAA ($mg.l^{-1}$)	1.0	1	0.1	0.2
NOA ($mg.l^{-1}$)	1.0	1	0.1	-
IAA ($mg.l^{-1}$)	1.0	1	0.1	-
P.V.P. ($g.l^{-1}$)	2.0	-	-	-

Abbreviation: BAP = 6-benzylaminopurine; IAA = indole acetic acid; IBA = indole-3-butyric acid; NAA = naphthalene acetic acid; K= kinetin; NOA = naphthoxy acetic acid; 2ip = 6(y-y-dimethylallylamino purine; P.V.P. = polyvinyl-pyrrolidone.

Effect of sucrose concentrations

This experiment was conducted on pear. The influence of different sucrose concentrations (0, 10, 20, 30, 40, 50, and 70 g/l) on shoot multiplication and the quality of shoots formation were observed in this experiment. Twenty-four explants for each treatment were cultured on medium (A) supplemented with 0.5 mg/l BAP. To prepare tissue specimens for histological study, tissue samples from the basal one cm of five shoots were harvested from each pear cultures of three treatments containing of 10, 30, and 50 g/l sucrose in the media. The tissue samples were fixed in FAA (formalin; acetic acid; alcohol) and embedded in paraffin wax. Transverse sections of 12 µm thick were cut and stained with ruthenium red and methylene blue, as the technique used by Locquin and Langeron [16, p. 352].

For all experiments, twenty-four explants of each treatment were used. Each experiment was conducted twice. Observations were made one month after culturing. All cultures of pear and quince were maintained at a constant temperature 22°± 1°C in a growth room with a 16h photoperiod by fluorescent tubes at 25 µ mol m^{-2} s^{-1}. The cultures of grape and date palm were maintained at a constant temperature 26°C ± 1°C in a growth room with 16h photoperiod by fluorescent tubes at 25 µ mol m^{-2} s^{-1}.

Results

Effect of BAP concentrations

BAP free medium was unfavorable for shoot multiplication. The shoot multiplication rate increased with increasing BAP concentrations in two material pear and quince (Table 2). The high concentrations of BAP increased the percentage of vitrified shoots (Table 2).

Table 2. Effect of different BAP concentrations on shoot multiplication and vitrification of quince and pear

BAP concentration mg/l	Quince		Pear	
	SM	VE %	SM	VE %
0	1	0	1	0
0.5	3.6	0	4	0
1	4.2	0	4.6	10
2	5.2	10	5.4	25
4	6.1	30	-	-

SM = Shoot multiplication rate; VE = % of vitreous explants.
Twenty-four explants per treatment, observed after one month of culture. Each number represents the mean of two experiments.

Effect of NH_4^+ concentrations

After one month of culture on MS medium containing a high concentrations of NH_4^+, the percentage of abnormal shoots was 20%. This problem of vitrification did not appeared on medium supplemented with low concentration of NH_4^+.

Effect of mineral salts solutions

In pear, the percentage of vitrified shoots on medium containing MS mineral salts was 45.8%, whereas it was 20.8% on medium containing Lepoivre mineral salts. The vitrified shoots were hyperhydric, translucid with yellow green color (Fig. 1, Photos 1 and 2).

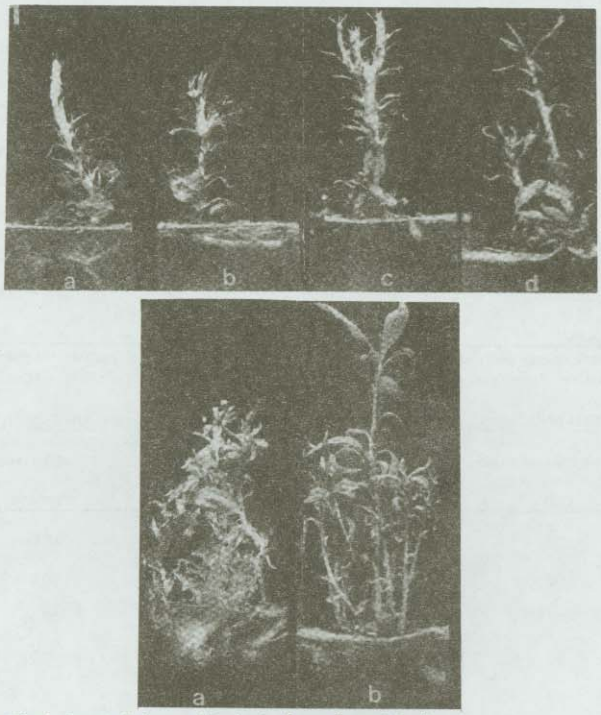

Fig. 1. Aspect of abnormality shoots of pear formed *in vitro*.
Photo 1. **a, b, c) abnormal shoots formed on medium containing high level of NH_4^+ concentration and d) normal explant as control.**
Photo 2. **a) abnormal shoots formed on medium containing high BAP concentration and b) normal shoots.**

In date palm, the aspect of vitrification was observed on medium containing MS mineral salts. The percentage of vitrification on medium containing Beauchesne [15] mineral salts was very low (Table 3).

The vitrified date palm tissues were hyperhydric, translucent; and were visually glassy yellow light-green or light-brown in color. The leaves were malformed and curled. It was possible to decrease or avoid vitrification by using Beauchesne mineral salts.

Effect of sucrose concentrations

After one month of culture on media at different sucrose concentrations, sucrose free-medium was unfavorable for shoot multiplication of pear. The best rate of multiplication was obtained on a medium containing 30g/l sucrose. Sucrose also affected the aspect of shoot formation. Furthermore, the percentage of vitrification was high on medium containing 10g/l sucrose; the vitreous plantlets decreased on medium with high concentration of sucrose (Table 4).

Table 3. Effect of mineral salts solution on shoot multiplication and vitrification of pear and date palm

Mineral salts solution	Plant	Shoot multiplication rate	% of Vitreous explants
Murashige and Skoog [14]	pear	5.1 a	45.8 a
Quoirin and Lepoivre [13]	pear	5.0 a	20.8 b
Murashige and Skoog	date palm	2.1 b	25.0 b
Beauchesna [15]	date palm	2.3 b	8.0 c

Twenty-four explants per treatment, observed after one month of culture. Each number represents the mean of two experiments. Treatments with the same letter are not significantly different at 5% level (Duncan's Test).

Table 4. Effect of different suucrose concentrations on shoot multiplication and vitrification of pear

Sucrose concentration (g/l)	Shoot multiplication rate	% of Vitreous explants
0	1.0 a	0.0 c
10	3.3 b	40.0 b
20	3.8 bc	20.0 a
30	5.0 d	0.0 c
40	4.3 cd	0.0 c
50	3.4 b	0.0 c
70	2.8 b	0.0 c

Twenty-four explants per treatment, observed after one month of culture. Each number represent the mean of two experiments. Treatment with the same letter are not significantly different at the 5% level (Duncan's Test).

The transverse sections showed a significant difference in the number of xylem layers formed on different media. A histological study was showed that shoots formed on media with 10, 30, and 50g/l of sucrose contained 4, 6 or 8 layers of xylem respectively (Fig. 2, Photo 1, 2 and 3). It means that the concentration of sucrose in the medium influenced the xylem formation as well as vitrification. The morphological differences between normal and vitrified shoots were reflected at the anatomical level.

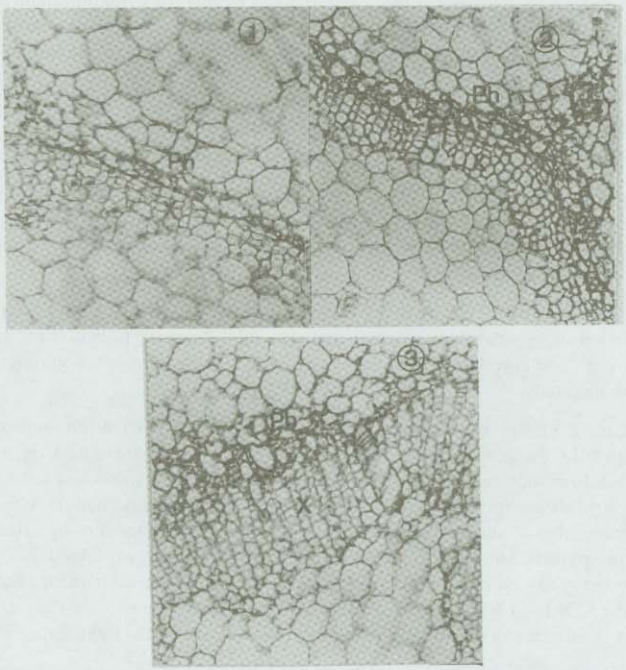

Fig. 2. Effect of sucrose concentrations on xylem formation and lignification of Pear shoots formed *in vitro*.
***Photo 1, 2, 3.* Transverse sections, showed the number of xylem layers of shoot formed on medium containing (10, 30, 50 g/l) of sucrose, respectively.**

Discussion and Conclusions

Micropropagation of fruit trees through tissue culture is very important for the production of large number of genetically uniform plants. Vitrification is one of the most important problems of *in vitro* plant propagation. The vitrified shoots cause a high damage *in vitro* plants production; moreover most of translucent shoots grew weakly and

lost their ability to propagate and developed roots *in vitro*; and most of them eventually died. When transferred to soil the plantlets did not survive even when kept under mist.

In pear and quince, vitreous shoots were observed at high BAP concentrations in the medium. The frequency of vitrification can be decreased by lowering the cytokinin concentration in the medium. Similar results were found by Debergh [4] in Malus and Prunus. The effect of cytokinin is less clear; it could be that the cytokinin-induced flush of mitotic activity coupled with a high matri potential and/or the test tubes atmosphere of 100%, relative humidity, result in the newly divided cells becoming overly turgid [17].

In grape, the frequency of abnormal shoots was increased at high NH concentration in the medium. The normal shoots were formed by using lower NH_4^+ in the medium. Our findings agree with the observations of Letouze and Daguin [18] on Prunus avium.

The percentage of vitreous explants in pear and date palm was increased by using MS salts solution in comparison with mineral salts of Beauchesne or Lepoivre. The MS minerals salts contains a high concentration of NH_4 NO_3, which was caused the formation of abnormal shoots in pear and date palm.

High concentration of NH, in the tissue influences the quantity of C/N, which affects the conversion of sugars to amino acids and a parallel decrease in cellulose synthesis [19]. This is a good correlation with the low level of lignification in the vitreous plants observed in this study.

In pear, a strong correlation was found between vitrification and sucrose concentration in the medium. The frequency of vitrification increased on medium containing a low sucrose concentration. A histological study showed a reduction in lignification in shoots formed on this medium. There is a good correlation between the lower concentration of sucrose in the medium, and the very low level of lignification in the vitreous plants. Moreover, the effect of sucrose can be explained by a good correlation between C/N ratio and vitrification. Low level of sucrose in the medium may decrease the C/N ratio which increased the formation of ethylene in vitreous explants. The excess of ethylene in the atmosphere of test tubes decreased the lignification [2].

Phan and Letouze [6] observed that phenolic compounds were more abundant in normal than the vitreous plants of Prunus avium; similar results were observed in Apple by Kevers, *et al.* [2]. Phenolic production is directly associated with C/N ratio, and they influence the phenomenon of lignification. This explains how the additions of PG and PZ to the culture media can help plants undergoing vitrification to return to a normal situation [19].

In conclusion, vitrification may be considered as a morphological response to non-wounding stress conditions, water logging, excess of NH_4^+ or cytokinin or low sucrose concentration. This stress could mediate a rapid endogenous ethylene burst. This gas decreases peroxidase activity which affects a low level of lignification [2].

According to the results of the different hypotheses tested to overcome vitrification *in vitro* propagation system of fruit trees, it could be concluded that the following factors influenced the vitrification.

In pear and quince, the use of low cytokinin concentration, mineral salts of Lepoivre, and high concentration of sucrose in the medium obtained the formation of normal shoots from vitreous plants. In grape, it can successfully avoid the formation of abnormal explants by lowering NH in the medium. In date palm, vitreous explants were not able to correct the disorder, and induce the formation of normal organs, but it can decrease the frequency of vitreous plant by using mineral salts of Beauchesne or other than MS and by adding activated charcoal to the medium.

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العوامل المؤثرة على ظاهرة الشفافية أثناء إكثار بعض أشجار الفاكهة بطريقة زراعة الأنسجة

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(سُلِّم في ٣٠ ذو الحجة ١٤١٥هـ؛ وقُبل للنشر في ١٣ شوال ١٤١٦هـ)

ملخص البحث. تعد ظاهرة الشفافية إحدى المشكلات الأساسية التي تعترض إكثار النباتات عن طريق زراعة الأنسجة، وقد لوحظت هذه الظاهرة الفسيولوجية أثناء التكاثر الخضري الدقيق للكمثرى، السفرجل، العنب والنخيل.

وقد أوضحت نتائج هذا البحث أن نسبة النموّات غير الطبيعية تزداد بازدياد تركيز هرمون البنزيل أمينوبيورين BAP وتركيز الأمونيوم NH_4 في الأوساط المغذّية. كما زادت نسبة النموّات الخضرية المصابة، أثناء طور الإكثار، عند استخدام المحلول المعدني موراشيجي وسكوج.

وقد تمّ الحصول على أنسجة سليمة بدءًا من أنسجة مصابة، في الكمثرى والسفرجل، عند استخدام المحلول المعدني لوبوافر Lepoivre، وتراكيز منخفضة من السيتوكينين BAP وتركيز مرتفع من السكروز. أما في النخيل فإنه لم يمكن الحصول على عيّنات سليمة من أنسجة مصابة بالشفافية، على الرغم من المحاولات الكثيرة التي أجريت، ولكن أمْكَنَ الحدّ من تشكل الأنسجة المصابة في النخيل باستخدام المحلول المعدني لبوشين Beauchesne.

كما أوضحت الدراسات التشريحية عن وجود علاقة وثيقة بين إصابة النموّات الخضرية بمرض الشفافية، مع درجة تخشب الأنسجة، حيث تزداد درجة التخشب في الأنسجة السليمة بالمقارنة مع درجة تخشب النباتات المصابة.

كما تمّ أيضًا مناقشة الدور الفسيولوجي لمختلف العوامل المؤثّرة في ظاهرة الشفافية.