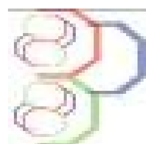

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Influence of growth regulators on callus induction from embryos of five citrus rootstocks

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ABSTRACT

Objective: The aim of this work is to develop a protocol for callus induction of Moroccan Citrus rootstocks.
Methodology and results: Callus cultures were initiated from embryos explants of five *Citrus* rootstocks (*Cleopatra mandarin*, *Rangpur lime*, *Citrus volkameriana*, *Trifoliate orange*, *Citrus aurantium*) on Murashige and Tucker (MT) and Tucker (MT) basal media. Different concentrations of growth regulators were tested in order to obtain the best callus formation: 2, 4-dichloro-phenoxyacetic acid (2, 4-D) in combination with benzylaminopurine (BAP) at five levels (0, 0.5, 1, 2 and 3mgL-1) were used in this study. It was found that growth regulator concentration had a significant effect on the callus induction, the callus growth and callus physical appearance. The highest frequency of the callus induction rate (100% and 83%) was observed with two combinations of 2, 4-D/BAP: 1 / 0, 5 and 2/1 (mgl-1). Medium containing only BAP (1mgl-1) resulted in the formation of large numbers of roots. Also, the callus induced on MT medium containing only 2, 4-D (1mgl-1) was brown in color and of low quality compared to that produced on MT media containing 2, 4-D/BAP. There was no callus formation on MT basal medium without growth regulators.
Also, the callogenesis depended on the genotype. It was maximal for *Cleopatra mandarin* followed by *Rangpur lime* and *Citrus volkameriana*, and then *Citrus aurantium* and *trifoliate orange*.
Conclusion and application: Passing through callus is paramount in vitro selection that plant tissue is an important source of genetic variability. The explants and exogenous phytohormones proved of paramount importance for the success of the callus and the formation of somatic embryos and their development.
Key words: Callus induction, Citrus rootstocks, 2, 4-D, BAP.

INTRODUCTION

Citrus is one of the most important commercial crops of the world valued for its juice and other by-products. According to UNCTAD in 2004, there were 140 citrus producing countries. In Morocco, citrus is considered one of the most important sectors of the national agriculture. It is the 3rd source of currency for the country (Exchange Office, 1999). This country occupies the second place after Spain in the Mediterranean region for the export of small citrus fruits followed by China and Turkey (FAO, 2003). On the social level, this sector is the main source of income for 10,000 farming families and provides, either directly (orchards) or indirectly (packing stations, ports and others) a total of 21 million days of work and provide more than 100,000 permanent jobs. However, the average yield of citrus remains low near 20 T/ he at national level (MADREF, 2003)

against more than 40 T/ he in some countries (Albrigo and Davis, 1994). Significant progress has been made in Morocco *citrus* breeding programs. Traditional breeding techniques have several limitations, such as access to a limited gene pool, crossing barriers, polyembryony, parthenocarpy, inefficient selection. Recent developments in biotechnology have opened opportunities to create new cultivars and rootstocks (Beloualy *et al,* 1993, Garcia-Agustin *et al,* 1995). The major objectives of citrus rootstock breeding include resistance and tolerance to biotic and environmental stress. Many studies were undertaken with the aim of controlling the techniques being able to lead in the genetic improvement of the selected citrus fruit species (Chakravarty *et al*, 1999). Establishment of an efficient callus induction protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaleda *et al*, 2006; Altaf *et al*, 2009). Citrus Embryos explants were most responsive to callus induction and proliferation (Alka, 2010). Thus, for biotechnological research on citrus, a reliable callus induction protocol using embryos is essential. The present study was undertaken with an objective to develop an efficient callus induction protocol which is a major prerequisite for *in vitro* plant regeneration system involving citrus rootstocks. And also induce somaclonal variation for the selection of new rootstocks tolerant to stress.

MATERIALS AND METHODS

Seed material: *Citrus* rootstocks seeds used in this study were obtained from the INRA, Regional Center of Kénitra, Morocco. Five rootstocks, planted in *citrus* collection, were used: Cleópatra mandarin, *Rangpur lime, Citrus volkameriana*, *Trifoliate orange*, *Citrus aurantium.*

Seed surface sterilization and explants: Seeds of each rootstock variety were extracted from ripe fruits. Under the laminar flow cabinet seeds were then immersed in ethanol 70% for 10 minutes, then in sodium hypochlorite solution 5% for10 minutes and finally washed three times by sterilized distilled water. After surface sterilization, embryos measuring approximately 2 mm in length were excised and cultured with embryonic face in contact with culture medium to promote callus formation in the apical pole of the embryo.

Effect of auxin in combination with cytokinine on callus induction: To assess the effect of auxin in combination with cytokinine, 2, 4-D was used in combination with BAP at five levels (0, 0.5, 1, 2 and 3 mg L-1). Basal media used on this study is the Murashige and Tucker (MT) and Tucker (MT) medium supplemented with 5% sucrose and 9% agar. The pH was adjusted to 5.5 before autoclaving at 121°C for 20 min. Callogenesis is initiated in the dark at 26 ± 1 C.

Statistical analysis: Five explants were cultured per Petri dish. All cultures were incubated for 120 days. The cultured Embryos were rated at weekly intervals. To set numerical values that represented both qualitative and quantitative growth, rating from 0 to 100% was developed (Figure 2, Table 1). All the experiments were repeated three times.Data obtained were subjected to analysis of variance 2 classification criteria (effect of rootstock, the effect of hormonal dose). This analysis was performed after normalization of data, expressed in percentage, the transformation Arc sin √%. The Newman-Keuls test was used for the comparison and ranking of averages. Letters (A, B, C, D) were used to indicate statistic differences between means.

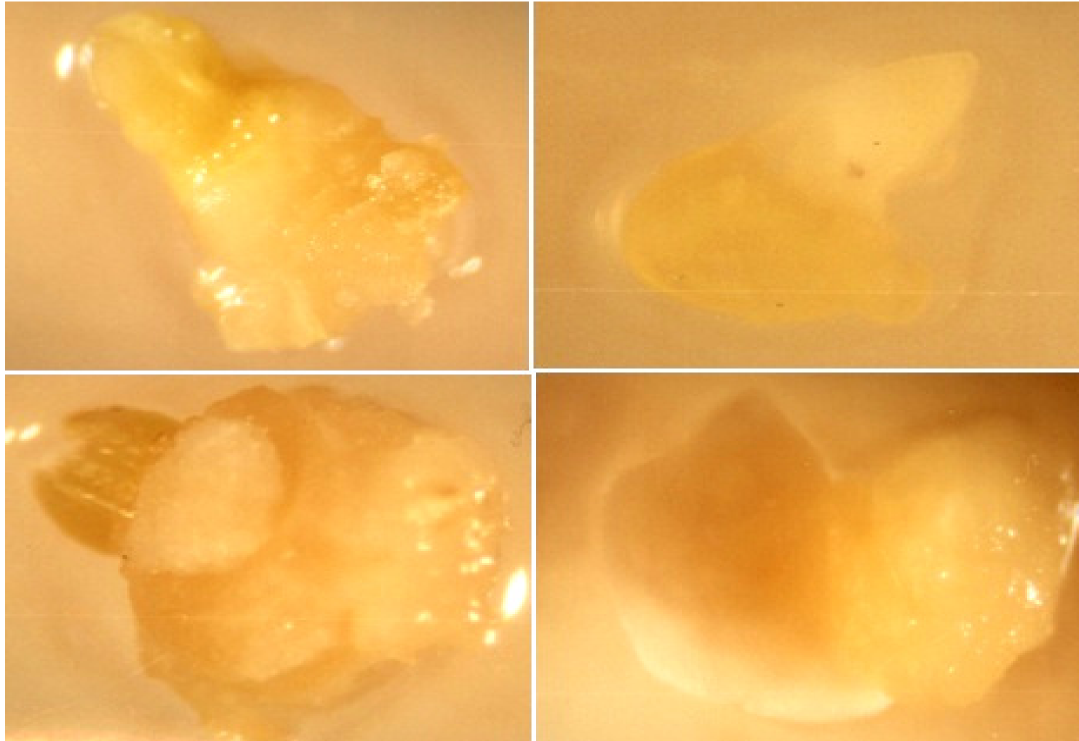

Figure 1: Transparent yellowish and friable callus induced from the apical pole of embryos rootstocks studied.

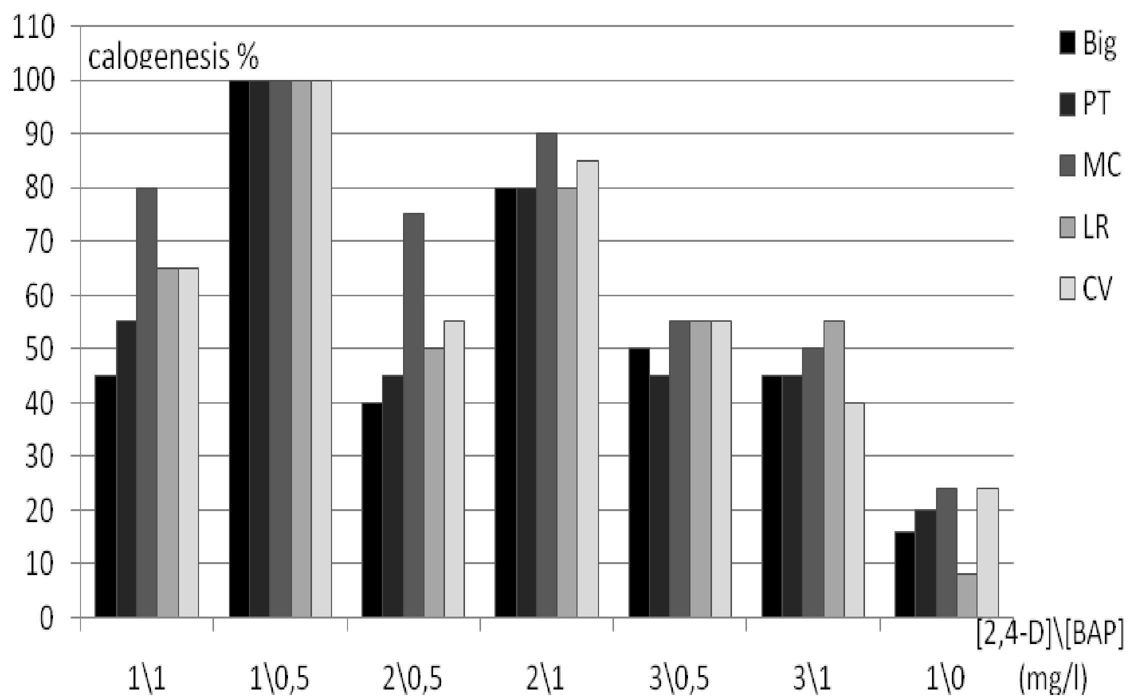

Figure 2: Callus Induction rate from embryos of five citrus rootstocks according the combinations of 2, 4-D and BAP.

Table 1: Effect of different combination of 2,4-D and BAP on callus induction from embryos explants cultured on MT medium in the dark at 26 ± 1 C.

2,4-D	BAP (mg/l)	Type of response	Callusing response (%)	Number of days for callus initiation	Capacity for growth	Consistence Appearance
0	0	-	-	-	-	-
1	0	Callus	18.4 **A**	20	Very low multiplication	Friable transparent
0	1	Rooting	-	-	-	-
1	0,5	Callus	100 **B**	8	High multiplication	Friable transparent and highly hydrated
1	1	Callus	62 **C**	19	low multiplication	Composite yellowish
2	0,5	Callus	53 **C D**	17	low multiplication	Composite orange
2	1	Callus	83 **B**	11	Good multiplication	Friable yellowish and highly hydrated
3	0,5	Callus	52 **C D**	19	Very low multiplication	Friable orange
3	1	Callus	47 **D**	29	low multiplication	Friable orange

RESULTS AND DISCUSSION

The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). Thus, biotechnological research on crops requires reliable callus induction and then efficient *in vitro* regeneration system. To study the combined effect of auxins and cytokinins on callus induction, five levels (0, 1, 0.5, 2 and 3 mgL-1) were used of 2, 4-D in combination with BAP. The result indicated that these combinations promoted the induction and growth of citrus callus. Callogenesis was observed after 8 to 29 days on all media tested (Figure 3), and the rate of induction varied significantly among rootstocks ($p<0.05$) and highly significant among hormonal doses ($P<0.01$) with no interaction between genotype and hormonal dose (table 2).

Table 2: Results of Analysis of Variance (ANOVA) for callus induction rate of five citrus rootstocks with different hormonal doses.

Source of variation	F observed	Pr > F
Rootstocks	1,14	0,3349 **
Hormonal dose	38,98	0,0001 ***
Rootstocks X Hormonal dose	0,36	0,9916 (ns)

** significant; *** highly significant; ns : non significant.

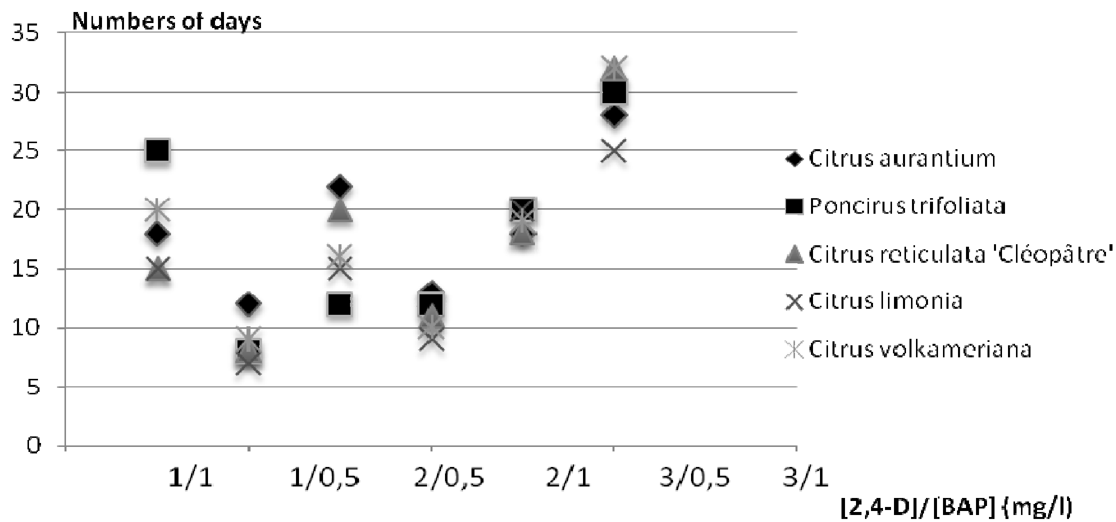

Figure 3: Numbers of days for callus induction from embryos of five citrus rootstocks according the combinations of 2, 4-D and BAP.

Also, culture of embryo, embryonic face in contact with the culture medium, promoted the proliferation of friable callus with a transparent yellowish nodular from the apical pole of the embryo (Figure 2) and this is for the five rootstocks and different combinations of 2, 4-D / BAP tested, although the presence of 2, 4-D alone at $1 mgL^{-1}$ is sufficient to callus initiation. This shows that the explants used were very sensitive to 2, 4-D and did not require high levels of auxin. The cell growth phase, related to the sensitivity to 2, 4-D, occurred in the first week of culture. However, the rate initiation was very low 18.4% in contrast to Sativa *et al.* (2010) who got a higher rate 96% in *Citrus jambhiri*. This shows that cell proliferation requires the presence of 2, 4-D as auxin essential for callogenesis. This acts as an inductive auxin signal to trigger the proliferative activity of explants. No reaction was observed in the control medium. This shows the important role of growth regulators. However, the callusing percentage, degree of callusing and callus appearance are auxin concentration dependant (Table 1). Essentially effects of auxins on citrus callus induction already have been reported (Huang *et al*, 2002, Savita *et al.,* 2010). Among different combinations evaluated for their effects on callogenesis, MT medium supplemented with 1 mg/L 2,4-D in combination with BAP at 0.5 mg/l supported highest rate of induction (100%), growth and calluses morphologically better developed (Figure 4, Pictures 1-5) for all the rootstocks studied, followed by 2 mg/L 2,4-D / 1 mg/L BAP (83%), 1 mg/L 2,4-D / 1 mg/L BAP (62%), 2 mg/L 2,4-D / 0,5 mg/L BAP (53%) and 3 mg/L 2,4-D / 1 mg/L BAP (47%) (Figure 2, Table 1). However, medium containing BAP (1 mg/L) alone resulted in the formation of large numbers of roots.

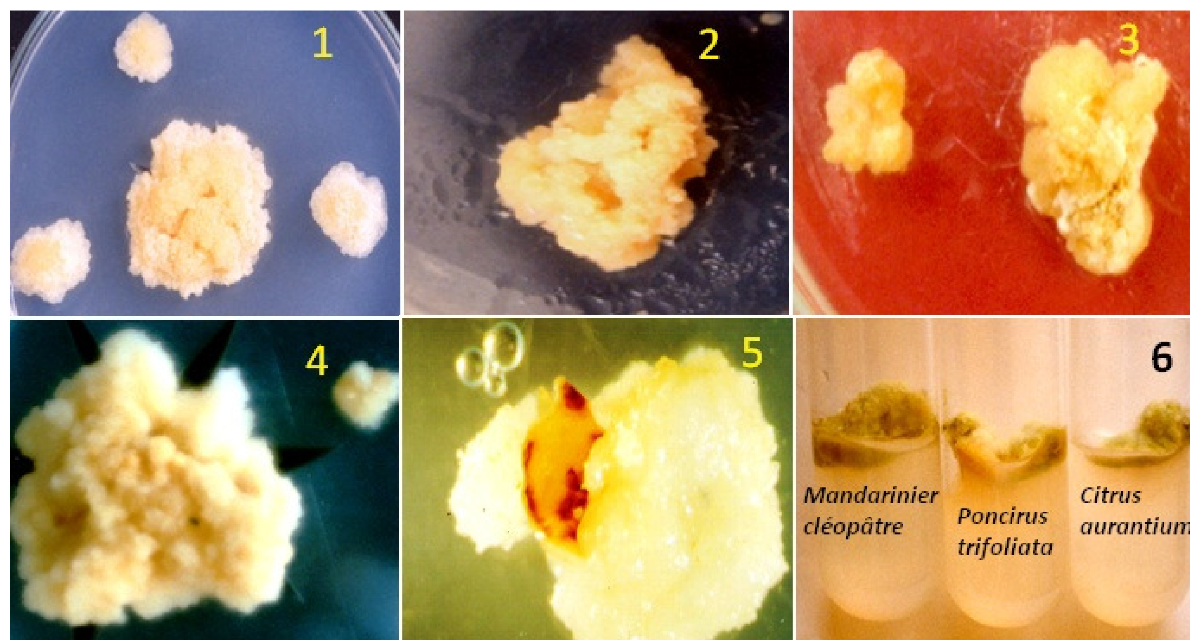

Figure 4: Pictures 1-5: Calluses obtained after 120 days in the dark from embryo culture on MT medium supplemented with 1/0, 5 (mg/l) of 2, 4-D/BAP. *Citrus aurantium* (Picture 1), Cleópatra mandarin (Picture 2), *Rangpur lime* (Picture 3), *Citrus volkameriana* (Picture 4) et *trifoliate orange* (Picture 5).
Picture 6: Callus transferred to light on MT medium supplemented with BAP alone (1mg/l).

Higher callogenesis rate is an important factor for establishing tissue culture and be particularly useful when there is a need to submit a uniform set of tissue to a treatment (Al-Kari *et al.*, 2001). It should be noted that the calluses obtained with combination 1/0 5 (mg/l) remained friable, transparent and highly hydrated and may be held for two years without any morphological change by planting every month in the dark for renewing the same medium (Figure 4, Pictures 1 to 5). A similar result on *Citrus acida* (Bipasha *et al.* 1999) shows that 2, 4-D in combination with BAP is one of the best combinations for induction and development of callus. This association suggests a synergistic and/or complementary effect of auxin and cytokinin, this latter further stimulates tissue sensitivity, particularly competent cells during callus phase. This effect has already been demonstrated in a large number of woody species such as Pistachio (Hafdi *et al.* 2000) and date palm (Asemota *et al.* 2007). Callus culture in the dark has significantly improved induction and growth of callus. The transfer to light in presence of BAP (1mg / l), blocked callogenesis launched photosynthesis and favored an early budding. This budding block remained even after transfers to other areas (MT+galactose (5%), MT+sucrose (5%), MT+ANA (1mg/l), MT+Kinetin (1mg/l)). This result shows the importance of pre-treatment callus by BAP for obtaining somatic embryos.

CONCLUSION

Development of an efficient tissue culture and plant regeneration protocol for citrus rootstocks is the first step towards the application of transgenic technology to improve citrus breeding and is, thus, the foundation of citrus biotechnology research program (Sharma *et al.*, 2009,). In this work, we have established callus induction protocol for Citrus rootstocks. This protocol will pave the way for the development of *in vitro* regeneration system for this cultivar and consequently will promote the application of plant tissue culture technology in the area of selection resistance, production of artificial seeds, and genetic transformation. Different callus obtained show no formation of somatic embryos. Extensive studies remain to be done to overcome the difficulties of obtaining embryogenesis and organogenesis from the callus to exploit somaclonal variation in the improvement of these rootstocks. Also, subsequent histological study should be developed to help detect the possible formation of embryogenic meristem cells.

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