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REGULAR ARTICLE

INFLUENCE OF MEDIUM AND GROWTH REGULATORS ON CALLOGENESIS OF QUINOA (*CHENOPODIUM QUINOA* WILLD.) AND EFFECT OF HYDROUS STRESS INDUCED BY P. E. G 6000 ON THE CALLUS

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ABSTRACT

The induction and growth of quinoa's callus depend on several factors, including the culture medium and the nature of the growth hormone and its dose. In effect, the best callogenesis rates were obtained with the media MS and B5 with respect to the media WHITE and KNOP the callogenesis is too low or zero. The best combination used was 0.2 BA+2.4 D give well-developed callus. To obtain water-stress resistant cell lines, the effect of water stress induced by polyethylene glycol (P. E. G 6000) on the growth, osmotic potential and metabolic parameter of Chenopodium quinoa callus was studied. Applied water stress showed a reduction in the growth of stressed callus compared to the control. The presence of PEG in the culture medium caused a decrease in the content of fresh matter as well as the dry matter content compared to the control. Water stress also significantly affected the water parameters of calluses. The chlorophyll a, b and carotenoids content decreased, but this decrease is not too pronounced.

Keywords: Callogenesis, *In vitro* culture, Drought stress, P. E. G, Quinoa

INTRODUCTION

The *in vitro* culture is a very recent technique since it was developed only at the beginning of this century by GAUTHRET [1], who was among the first to cultivate tissues, coming from cambial cells of different trees (poplar, maple, willow), on agar medium under aseptic conditions. Today, under the term "*in vitro* culture" is hiding much diversified fields and techniques, which strongly developed and specialized since thirty years. In the vegetable field, *in vitro* culture methods apply to a very diversified material (protoplasts, tissues, organs, whole plants), to achieve oriented objectives, either towards research or towards industrial production [2].

On the other hand, plants can be regenerated from callus tissues of various explants by dedifferentiation prompted by exogenous growth regulators. Regeneration from callus is by organogenesis or somatic embryogenesis [3]. The present study was conducted with an aim of finding the influence of medium and growth regulators on callogenesis of quinoa (*Chenopodium quinoa* Willd.) and effect of hydrous stress induced by P. E. G 6000 on the callus.

MATERIALS AND METHODS

Experimental details

The experiments were carried out in the *in vitro* culture unit at the Arid Area Institute (IRA), located at el FJE Medenine (33 °03' N; 10 °38' E) in south-east Tunisia. The quinoa's calluses are initiated from stems (fragments of internodes; 5 cm long) of the cultivar Q-37 originated from Chile at the rate of ten explants × three repetitions per test.

For the sterilization of the plant material, we applied the most used method, which is proposed by GAUTHRET [4], by putting it in a solution of sodium hypochlorite 50% (NaClO) for 30 min, followed by five rinses with sterile distilled water during five minutes each, to remove traces of bleach. Then we have put fragments, in 70% alcohol for five minutes, five rinsing with sterile water of five minutes each one to remove all traces of alcohol. This is done under a laminar flow hood.

The transplanting material (scalpels, tongs, petri-dishes, etc.) is cleaned and then autoclaved in a vapor phase at 120°C for 20 min. The culture mediums are also sterilized by autoclaving. The pH's medium is adjustets to 5,5-5,8 with a solution of NaOH (1N).

During the manipulation, each tool is rinsed in 70 alcohol. Finally, the cultures are placed in an air-conditioned chamber at 25±1°C, with "Phillips-40 W" tubes, guaranteeing an illumination of 2000-2500lux. The photoperiod is 16 h of light a day.

In this study we try to find the most favorable nutritional conditions for the callogenesis of quinoa's explants. For this, we have chosen the following mediums: MS [5], B5 [6], KNOP and WHITE [7] (table 1). The main constituents of these

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media are ionized water and mineral salts, which are divided into two groups: macroelements (N, P, K, S, Mg, Ca) and microelements (Fe, B, Mn, Zn, Cu, Co, Mo, I). The source of carbon is sucrose and in these media is found vitamins, amino acids and gelling agent.

The culture mediums are enriched by growth regulators: auxins (2,4-D: 2,4-dichlorophenoxyacetic acid, ANA: 1-naphthaleneacetic acid) and cytokines (BA: 6-benzyladenine). They are brought alone or in combination in culture media.

After two months of cultivation the following measures were made:

• Percentage of callogenesis with simple counting;

• Diameter of the calluses using a sliding caliper;

• Color and aspect of calluses.

Effect of water stress on callus

To test the effect of hydrous stress on callogeninis of quinoa's tissues, we have used Polyethylene Glycol 6000 (P. E. G) which is a polymer of chemical formula $COCH_2(C_2H_4O)_n$. The various levels of water potential were obtained by the formula established by MICHEL and KAUFMAN [8], this equation relates the hydric potential (ΨH), the concentration of P. E. G and the temperature:

$\Psi H=-(1,18*10^{-2})\ C-(1,18*10^{-4})\ C^2+(2,67*10^{-4})\ CT+8,39*10^{-7}\ C^2\ T$

With: ΨH: water potential (bar);

T: incubation temperature (C °);

C: concentration of P. E. G 6000 (g/l).

The solutions of P. E. G prepared corresponds to the values of the following water potentials: 0 bar (T0);-1 bar (T1);-2 bars (T2).

To avoid the influence of the effect of the medium on the results, the calluses initiated from internodes are growing on the medium MS+0.2 mg/l BA with ten calus × three repetitions for each treatment.

In order to detect the effect of water stress on the morphological, hydric and metabolic characters of calluses; various parameters were measured after two months of culture (table 2).

Data analysis

All data were statistically elaborated using analysis of variance (ANOVA), followed by means separation using S-N-K's multiple range t-test at $P<0,05$. All calculations were performed with the help of the PASW statistics 18.

RESULTS

Effect of culture medium and hormonal composition on callogenesis

The effect of different concentrations of cytokinine BA combined to auxin (2,4D/ANA) and added to the Murashige and Skoog (MS), WHITE, the B5 and the KNOP mediums on induction of callus was studied. The results are presented in table 3 and fig. 1, 2, 3 and 4).

Table 1: Composition of MS [5], B5 [6], KNOOP and WHITE [7] mediums

Medium components (mg. l^{-1})	MS	B5	KNOOP	WHITE
Macronutrients				
NH_4NO_3	1650			
KNO_3	1900	2500	250	80
$CaCl_2.2H_2O$	440	150		
$MgSO_4.7H_2O$	370	250	250	737
KH_2PO_4	170		250	19
$(NH_4)2SO^4$		134		
$NaH_2PO_4.\ H_2O$		150		
$CaNO_3.4H_2O$			1000	288
Na2SO4				200
KCl				645
Micronutrients				
KI	0,83	0,75	0,01	0,75
H_3BO_3	6,3	3	1	1,5
$MnSO_4.4H_2O$			1	6,7
$MnSO_4.\ H_2O$	22,3	10	0,1	
$ZnSO_4.4H_2O$			1	2,2
$ZnSO_4.7H_2O$	8,6	2		
$Na_2MoO_4.2H_2O$	0,25	0,25		
$CuSO_4.5H_2O$	0,025	0,025	0,03	
$CoCl_2.6H_2O$	0,025	0,0125		
Na_2EDTA	37,3	37,3		
$FeSO_4.7H_2O$	27,8	27,8		
$Fe_2(SO_4)_3$				2,5
Vitamins				
Inositol	100	1	1	1
Glycine	0,2		20	20
Thiamine HCl	0,1	100	1	1
Pyridoxine HCl	0,5	10	5	5
Nicotinic acid	0,5	1	5	5
Sugar				
Sucrose	3000	2000	3000	3000
Gelling agent				
Agar-agar (g/l)	8	8	8	8

Table 2: Various parameters measured to study the effect of hydrous stress on callus

Morphological parameters	Water parameters	Physiological parameters
Callus diameter (mm)	WC (ml g^{-1}DM)= (FW-DW)/DW. [9]	Chlo a (µg. g^{-1})= $12.7A_{663}$-$2.69A_{647}$ Chlo b (µg. g^{-1}) = $22.9A_{647}$-$4.68A_{663}$ [10]
Fresh weight (FW) (g) Dry weight (DW) (g)	RWC (%) = ((FW-DW)/(TW-DW)) × 100. [11]	Car (µg. g^{-1}) = 5 A_{470+}2.846 A_{663}-$14.876A_{647}$ [10]

WC: water content; RWC: relative water content; DWS: Deficiency of water saturation; TW: turgor weight; Chlo a: chlorophyll a content; Chlo b: chlorophyll b content; Car: carotenoids.

Table 3: Effect of medium and hormonal composition on callogenesis's parameters

		Hormonal composition								
	Medium	**0,1 BA**	**0,2 BA**	**0,5 BA**	**0,2BA+0,1 (2,4D)**	**0,2BA +0,2 (2,4D)**	**0,2BA +0,5 (2,4D)**	**0,2B +0,1 ANA**	**0,2BA+0,2 ANA**	**0,2BA+0,5 ANA**
Induction (%)	MS	90	100	90	100	100	100	90	80	70
	B5	100	100	100	80	90	100	60	60	80
	WHITE	0	0	10	0	0	0	0	0	0
	KNOP	0	0	0	40	30	10	30	0	20
Callus Ø (mm)	MS	$1,09^{a}$	$1,53^{abc}$	$1,5^{abc}$	$1,79^{bc}$	$1,57^{abc}$	$1,95^{c}$	$1,17^{abc}$	$1,34^{abc}$	1^{a}
	B5	$1,39^{ab}$	$1,71^{ab}$	$1,4^{ab}$	$1,43^{ab}$	$2,01^{b}$	$1,81^{ab}$	$1,28^{a}$	$0,92^{ab}$	$1,46^{ab}$
	WHITE	-	-	1^{a}	-	-	-	-	-	-
	KNOP	-	-	-	$1,32^{a}$	$1,35^{a}$	$1,3^{a}$	$1,15^{a}$	-	$1,37^{a}$
Aspect	MS	Fri	Fri	Fri	Fri	Fri	Fri	Com	Fri	Com
	B5	Com	Com	Com	Fri/ Com	Com	Fri	Com	Com	Fri/ Com
	WHITE	-	-	Com	-	-	-	-	-	-
	KNOP	-	-	-	Fri	Fri	Fri	Com	-	Com
Color	MS	Gr	Gr	Gr	Dgr	Dgr	Dgr/ Lgr	Dgr	Dgr	Lgr
	B5	Red	Red	Red	Red	Red	Red/Dgr	Lgr	Dgr/ Lgr	Lgr
	WHITE	-	-	Lgr	-	-	-	-	-	-
	KNOP	-	-	-	Lgr	Lgr	Lgr	Lgr	-	Lgr

Fri: friable; Com: compact; Gr: green; Dgr: Dark green; Lgr: light green, (a, b, c: for numbers followed by different letters, the difference is very highly significant (p≤0,001))

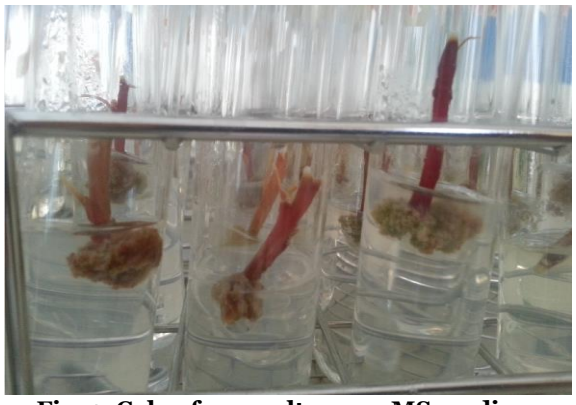

Fig. 1: Calus from culture on MS medium

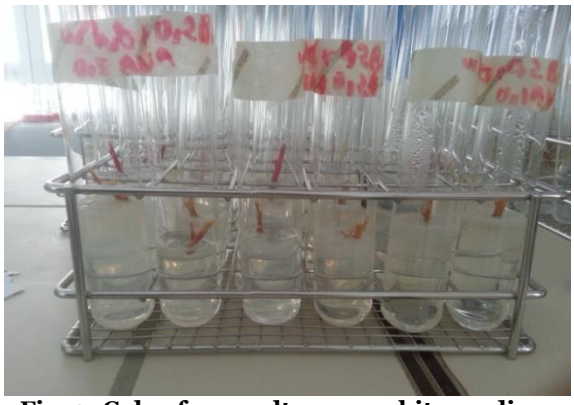

Fig. 2: Calus from culture on white medium

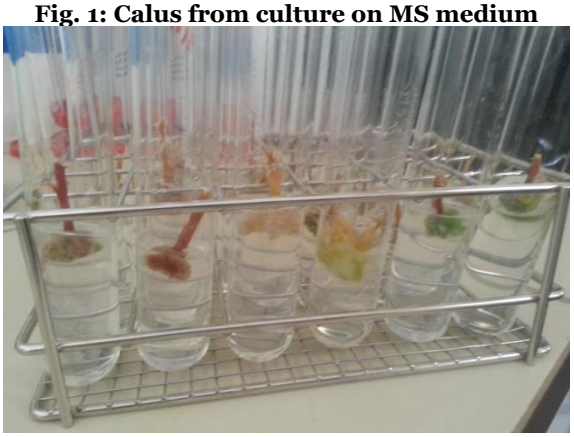

Fig. 3: Calus from culture on B5 medium

Fig. 4: Calus from culture on KNOP medium

The addition of BA on MS and B5 medium has significantly improved callogenesis rates which are well ranging between 90% and 100% in the presence of low doses. On the other hand, an improvement proportional to the BA concentration of the callus diameter is recorded ($p<0,05$) which has reached the maximum for the 0,5 mg/l concentration of 1,55 cm. It has also been noted that the presence of 2,4D in combination with BA at a dose of 0,5 mg/l has favored the appearance of roots in certain callus with MS medium (fig. 5).

Effect of hydrous stress on callogenesis

There is a difference between the average diameters of the control calluses and the stressed ones, this difference is accentuated with the concentration of the medium with P. E. G. A reduction of 26% and 60%, respectively, for the water stress-1bar and -2bars (fig. 6).

Fig. 5: Calus with roots

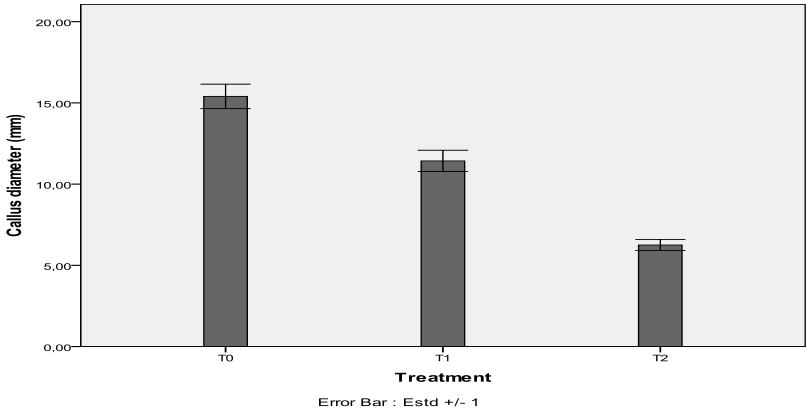

Fig. 6: Effect of water stress on callus diameter

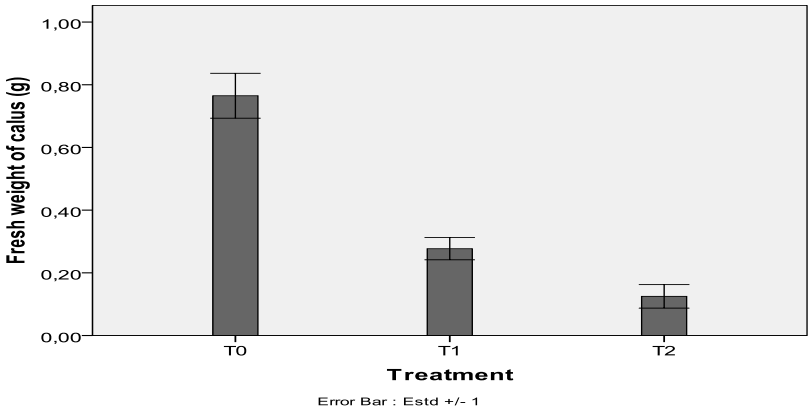

Fig. 7: Effect of water stress on callus's fresh weight

The analysis of the results indicates that the production of fresh matter is clearly affected by water restriction. During the culture, the difference is in favor of the control and the reduction is highly significant ($p<0.01$) of the order of 70% for-1bar and 84% for-2 bars (fig. 7).

The analysis of this fig. shows that, in the absence of water stress, calluses have the highest dry matter content (0,064g), whereas in the presence of P. E. G 6000, a highly significant decrease was recorded for stressed ones ($p<0.01$) (fig. 8).

Water stress has negatively affected the moisture content, which is further reduced when P. E. G concentrations are high. This is confirmed by the ANOVA data ($p<0,01$). A maximal average (13 ml/g) was registered under unstressed medium. The water content is reduced to 70% under the water regime T2 (fig. 9).

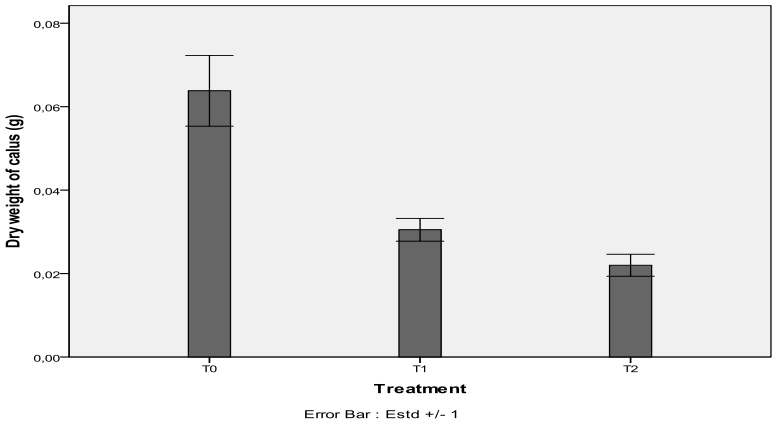

Fig. 8: Effect of water stress on callus's dry weight

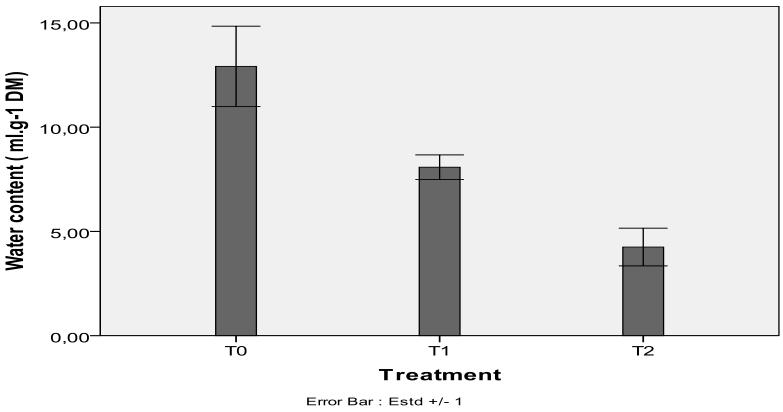

Fig. 9: Effect of water stress on callus's water content

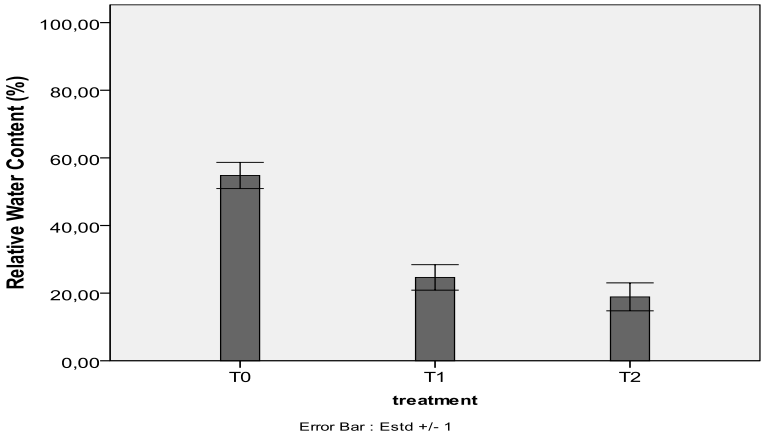

Fig. 10: Effect of water stress on callus's relative water content

The results of this fig. show that RWC was significantly affected calluses subjected to water stress (P<0.01). Under (-2) bars regime, a reduction of 55% was registered in comparison with the control calluses.

The chlorophyll content showed a significant (p<0.01) decrease. The response of calluses to water stress is proportional to the intensity of the water deficit (fig. 11). This decrease does not lead to 13% for T1 treatment, while it is more pronounced under T2 stress in the order of 42% compared to controls.

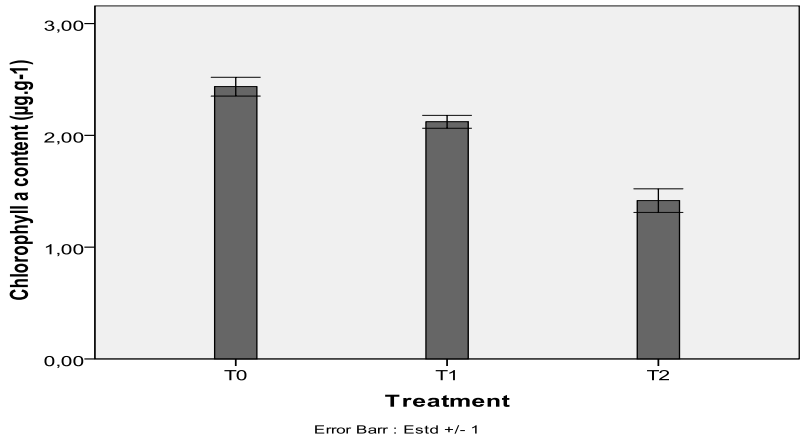

Fig. 11: Effect of water stress on callus's chlorophyll a content

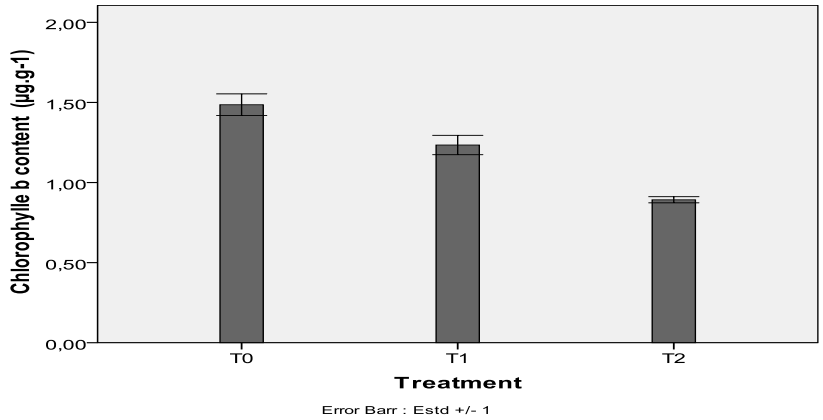

Fig. 12: Effect of water stress on callus's chlorophyll b content

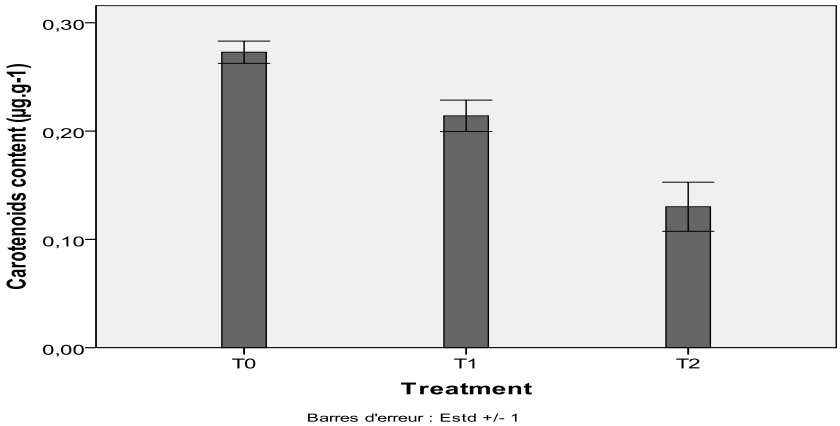

Fig. 13: Effect of water stress on callus's carotenoids content

The examination of the fig. 12 shows that the chlorophyll b content is closely related to the applied water regime. Analysis of the data shows a highly significant reduction ($p<0,01$). In fact the recorded contents are 1,48±0,07 µg. g^{-1}; 1,23±0,07µg. g^{-1} and 0,89±0,07µg. g^{-1} respectively for T0, T1 and T2.

Statistical analyzes of callus's carotenoid accumulation showed significant differences ($P>0,01$) between treatments. A 50% decrease was recorded in the most stressed calluses (0,13µg. g^{-1}) (fig. 13).

DISCUSSION

The effect of medium and growth regulators on callogenesis

The monitoring of callogenesis rate and callus's diameters revealed significant differences between the culture mediums favoring both MS and B5 ($p<0,01$). This difference can be explained by the effect of the variation of mineral elements that constitute them. The difference is essentially due to the nitrogen and potassium contents [12]. Taking into account the nitrophilic tendency of most Chenopodiaceae [13], the presence of sufficient nitrate in MS (1900 mg/l) and B5 (2500 mg/l) medium favored cellular development of quinoa's explants.

Contrariwise, the KNOP and WHITE mediums are poor of potassium nitrate (KNO_3) that's why they showed very low levels of callogenesis. Similar results have been proven by Radosvich and Paupardin [14] with axillary budding of quinoa proving that for a potassium nitrate content equal to 2700 mg/l, the development of axillary buds had increased.

Also, the beneficial effect of K and Mg ions has been reported by several authors [15, 16, 17]. These ions are also known to promote plant growth. CABECHE [18] mentioned the very important role of the nitrogen/carbohydrate ratio in the control of the biosynthesis of growth regulators in undifferentiated tissues. The callus grown on the KNOP medium, marked by iron deficiency, are characterized by a light green color, which explains why exogenous iron can be involved in the synthesis of the structural elements of chloroplasts, thus directly influencing the synthesis of chlorophyll [14]. It can thus be said that the composition of the *in vitro* culture base medium plays a very important role in the callogenesis of the quinoa explants. The effect of the medium results from the whole of the interactions of the different elements which compose it. Some of them stimulate *in vitro* development processes, while others have less influence on development [12, 19, 20]. On the other hand, the influence of cytokins on callogenesis is poorly documented. Some authors [21] consider that the addition of cytokins in the induction medium causes, at low or high doses, browning of the tissues which is harmful to the formation of callus. For the coconut tree, Verdeil *et al.* [22] notes that they are not essential for callogenesis. These results are contrary to our observations. Indeed, for quinoa, the presence of cytokinin BA in culture media MS and B5 gave very high callogenesis rates of the order of 100%. Our results are in line with those of Branton and Blakes [23], which show that cytokins sometimes stimulate callogenesis.

During the experience, we had also noticed that development of callus and their consistency varied with the hormonal composition used. Callus formed on MS and B5 media with the presence of 2,4D+0,2 mg/l BA have significant development of diameter and they are friable, similar results have been demonstrated by the work of EISA *et al.* [24] on quinoa. Callus formed with the presence of ANA are smaller and compact. An improvement in callogenesis rates is observed by the addition of auxin in the culture medium. Several studies have showed that the use of auxin induces triggering or induction of callus in a large number of botanical species: the oil palm [25]; *Atriplex halimus* [26, 27, 28]; banana and plantain [29]; *Phoenix dactylifera* L. [30]. One of the functions of auxin is to stimulate the mitotic activity of the cambial tissues, a property widely exploited in tissue culture *in vitro* [31, 32].

Other studies have demonstrated the positive effect of combining cytokinin (BAP or kinetin) with auxin (AIA, ANA, AIB). According to Ramirez *et al.* [33], Mtimet [34], Lemhamdi [35], and Mhatre *et al.* [36], the combination of an auxin with a cytokin promotes an increase in the number of shoots per plant. For this reason, Chatibi *et al.* [37] and Chatibi [38] reported that addition of growth regulators is essential for *in vitro* multiplication of ligneous plants and that axillary bud neoformation varies with the nature and combination of growth regulators used. Masmoudi [39], working on the callogenesis of the date palm, variety Arichi, has also shown that, although it is essential, a toxic effect of high concentration of 2,4-D (10 mg/l) on callus manifested by an early senescence of explants.

Finally, the growth regulators used in these experiments (BA, 2,4-D and ANA) and their concentrations are able to modify the "register" of cell totipotency and the externalization of the potential for organogenesis in the explants of quinoa.

The effect of hydrous stress on callogenesis

The addition of increasing concentrations of P. E. G 6000 to MS medium induced a very significant diminution of the morphological, water and physiological parameters of callus. Indeed, this reduction is all the more important as the concentration of P. E. G increases. These results show that P. E. G 6000 can be used to create *in vitro* water stress. Its role as a factor that induces water stress has been proved by different authors [40, 41, 42].

High concentrations of P. E. G 6000 severely inhibited callus growth. Dehydration of stressed callus may be primarily due to the direct effect of P. E. G 6000 and secondly to osmotic adjustment by accumulation of organic solutes and minerals [43]. Such behavior has been frequently reported in woody or herbaceous species and it's considered as an adaptive response to drought [44]. When stress is not tolerated by the plant, both fresh and dry yields are reduced, this has been shown also by the work of Trought and Drew [45].

The analysis of the water parameters of the callus, can describe in a global way the water status in response to the hydric stress undergone, as well as to give the level of turgor at the cellular level [46]. In our study, the effect of PEG induced a substantial decrease in water content (WC) and relative water content (RWC). This reduction is relatively small in comparison with the high concentration of P. E. G in the culture medium. Our results are consistent with those reported in *Sesuvium portulacastrum* [47] and *Aeluropus lagopidesi* [48].

The work of Malin *et al.* [49] and Diaz-Perez *et al.* [50] suggest that genotypes achieve high RWC, despite water stress, are tolerant.

Also, hydrous stress had a negative effect on the levels of chlorophyll pigments. There was a gradual decrease, proportional to the intensity of the water stress, of chlorophyll a, b and carotenoid contents. Our results corroborate those of Tahri *et al.* [51] and Scheirs and Bruyn [52].

It should be noted that the presence of selective agents in the medium may increase the probability of obtaining tolerant plants [53]. Tolerant plants have been obtained after *in vitro* selection by P. E. G of durum wheat [54, 55], rice [56, 57] and sorghum [58] and leading to genetic drift [59].

CONCLUSION

The objectives of this work were to develop techniques of micro propagation by callogenesis of chenopodium quinoa as well as the determination of the behavior of the callus against water stress by addition of P. E. G. At the end of this work the following conclusions can be drawn:

-the most favorable mediums for the callogenesis of quinoa explants are MS and B5;

-the best hormonal combination giving the best results is 0,2BA+0,5(2,4D);

-the effect of water stress on callus is reflected by a decrease in diameter, fresh and dry matter content, water and chlorophyll pigment content; this reaction is a mechanism of resistance to drought stress of quinoa at the cellular level.

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REFERENCES

1. Gautheret R. J. 1934. La culture de tissus cambial. C. *R. Acad. Sci.* 198, 2195-2196.
2. Jay-Allemand C., Capelli P. et Cornu D. 1992. Root development of *in vitro* hybrid walnut microcuttings in a vermiculite containing gelrite medium. *Scientia Horticulturae*, 51, 335-342.
3. Mathur J. and Koncz C. 1998. Callus Culture and Regeneration. *Methods in Molecular Biology*. 82, 1-5.
4. Gautheret RJ. 1939. La possibilité de réaliser la culture indéfinie des tissus de tubercules de carotte. *Acad. Sci.* Paris, 208, 118-120.
5. Murashigue,T. and F Skoog., 1962. A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissus Cultures. *Physiologia Plantarum,* 15:473-497.
6. Gamborg Ol, Miller R, Ojiama K.,1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res*; 50:151-158.
7. Gautheret R. J. 1959. La culture des tissus végétaux technique et réalisation. Maison et cie. 884p.
8. Michael B. E. et Kaufmann M. R. 1973. The osmotic potential of polyethylene glycol 6000. *Plant physiology*, 51:914-916.
9. Atmani Rochdi J., Lemsellek A., Bousarhal A. and Abdellatif R. 2005. Evaluation sous serre de la tolérance à la salinité de quelques porte-greffes d'agrumes: *Citrus aurantium* et deux hybrides de *Poncirus trifoliata* (*Poncirus×Citrus sinensis* et *Poncirus* ×Mandarinier sunki). *Biotechnol. Agron. Soc. Environ*.9, 65-73.
10. Arnon, D. I. 1949. Coper enzymes in isolated chloroplasts. *Plant Physiol*. 24:1-15.
11. Clarck J. M and Mac-Caig T. N. 1982. Excised leaf water relation capability as an indicator of drought resistance of Triticum genotypes. *Canada Journal Plant Science* 62, 571-576.
12. Brhadda N, Abousalim A, Walali Loudiyi D, Benali D. 2003. Effet du milieu de culture sur le microbouturage de l'olivier (*Olea europeae L.*) cv. Picholine Marocaine. *Biotechnol. Agron. Soc. Environ*. 7, 177p.
13. Rea J., Tapia M. E., Mujica A. 1979. Prácticas agronómicas. *La Quinua y la Kañiwa, cultivos andinos. Tapia, M. E., Gandarillas, H., Alandia, S., Cardozo, A. et Mujica, A. (eds). CIID-IICA. Bogota, Colombia*. 83-120.
14. Radosevich M. and Paupardin C. 1985. Vegetative propagation of Chenopodium Quinoa by shoot culture. *Amer. J. Bot*. 72: pp278-283.
15. David HK. Isemukali K., David A., 1978. Obtention de plants de pin maritim (*Pinus pinaster Soland.*) à partir de brachyblastes ou d'apex caulinaires de très jeunes sujets cultivés *in vitro*. *C. R. Acad. Sci.* 287, 245-248.
16. Bornmun C. H., 1983. Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. *Physiol. Plant*. 57, 5-16.
17. Margara J., 1978. Mise au point d'une gamme de milieux minéraux pour les conditions de la culture *in vitro*. *C. R. Acad. Sci*. 8, 654p.
18. Cabeche M., 1987. Nitrogène carbohydrate and zinc requirements for the efficient induction of shoot morphogenesis from protoplast-derived colonies of *Nicotiana plumbaginifolia*. Plant Cell. *Tissue Org. Cult*. 8, 197-206pp.
19. Rugini E., Caricato G., 1995. Somatic embryogenesis and recovery from mature tissues of olive cultivars (*Olea europaea L.*) "Canino" and "Moraiolo". *Plant Cell Rep*. 14, 257-260pp.
20. Grigoriadou K, Vasilakakis M, Eleftheriou E. P.,2002. *In vitro* propagation of the Greek olive cultivar Chondrolia C halkidikis. *Plant Cell, Tissue Org. Cult*.7 1,. 47-54.
21. Paranjothy, K. and Othman R., 1982. ln vitro propagation of oil palm 5th IntI. *Congo Plant Tissue and Cell Culture*: 755-756.
22. Verdeil J. L., Buffard-Morel, J. and Pannetier, C., 1989. Somatic embryogenesis of coconut (*Cocos nucifera L.)* from leaf and inflorescence tissues: research findings and prospects. *Oléagineux*, 44:403-411.
23. Branton, R. L. and Blake, J., 1984. Clonal propagation of coconut palm. *Proc. Int. Conf. Cocoa coconuts*, 46:1-9.
24. Eisa S., Koyro H. W., Kogel K. H and Imani J. 2005. Induction of somatic embryogenesis in cultured cells of Chenopodium quinoa. *Plant cell, Tissue and Organ* 81:pp243-246.
25. Ahée J. Arthuis P., Cas G., Duval Y., Guénin G., Hanower J., Hanower P., Lievoux D., Lioret C., Malaurie B., Pannetier C., Raillot D., Varechon C. et Zucherman L. 1981. Oléagineux,Vol. 36 .
26. Benrebiha, F. 1998. Etude de la callogénèse et des facteurs d'induction de l'embryogenèse cher *Atriplex halimus* L. In « Etude de la biodiversité biologique de l'*Atriplex halimus* L. pour le repérage *in vivo* et *in vitro* d'individus résistants à des conditions extrêmes du milieu et constitution de clones ». In P. Dutuit (Eds) Projet européen SDT3. Rapport final.

27. Benrebiha, F. 2005. Etude de différents milieux de culture, de substances de croissance et de salinité sur la morphogenèse de *l'Atriplex halimus* L. INA El Harrach. Algérie. Thèse 139p.
28. Cabello, A. et Bravo, C. 1998. Etudes de microbouturage sur *Atriplex halimus* L. In : étude de la biodiversité biologique de *l'Atriplex halimus* L. pour le repérage *in vivo* et *in vitro* d'individus résistants à des conditions extrêmes du milieu et constitution de clones. In P. Dutuit (Eds) Projet européen SDT3 (1994-1998) Rapport final.
29. Strosse H., Domergue R., Panis B., Escalant J. V. et Côte F. 2003. Suspensions cellulaires embryogènes de bananiers et bananiers plantain (*A. Vézina* et *C. Picq,* eds). Guides techniques INIBAP8. Réseau international pour l'amélioration de la banane et de la banane plantain, Montpellier, France.
30. Sané D., Aberlenc-Bertossi F., Gassama-Dia Y. K., Sagna M., Trouslot M. F., Duval Y. and Borgel A. 2006. Histological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera* L.). *Ann. Bot. 98:* 301-308.
31. Lafon J. P., Tharaud-Prayer C. et Levy. 1987. Biologie des plantes cultivées: Physiologie du développement génétique et amélioration. *Lavoisier,* 172p.
32. Augé R. 1989. Les phénomènes physiologiques liés à la réalisation des cultures *in vitro*. La culture *in vitro* et ses applications horticoles. *Lavoisier*, 7-29.
33. Ramirez-Molgan R., Borodanenko A., Barrera-Guerra J. K. et Ochoa-Alejo N. 2001. Shoot number and shoot size as affected by growth regulators *in vitro* culture of *Spathiphyllum floribundum L. Sci Hort,* 89:277-236.
34. Mtimet M. 2007. Culture *in vitro* du cultivar d'olivier 'Chemlali Djerba': micropropagation, callogenèse et comportement des cals vis à vis des stress abiotiques. Institut National Agronomique de Tunisie, p119.
35. Lemhamdi A. 2006. Micropropagation d'une espèce industrielle cultivée dans le sud tunisien: le Henné (*Lawsonia inermis L.*). Mastère en génétique et bioressources. Fac. Sci. Tunis, 79 p.
36. Mhatre M., Salunkhe C. K. et Rao P. S., 2000. Micropropagation of *Vitis vinifera* L.: towards an improved protocol. *Sci Horti,* 84:357-363.
37. Chatibi A, Kchouk M. L., Zemni H. et Ghorbel. A. 1996. Organogenèse *in vitro* du pistachier *(Pistacia vera* L.) CV. Mateur à partir de feuilles d'embryons zygotiques, 3p.
38. Chatibi A.1999. Les différentes potentialités de régénération *in vitro* du pistachier (*Pistacia vera* L.) CV. Mateur. Sc. Biol. Fac. Sc. Tunis, Thèse,179p.
39. Masmoudi R. 1999. L'embryogenèse somatique chez le Palmier Dattier (*Phoenix dactylifera L.*): induction et maintien des structures embryogènes, caractérisation biochimique. Faculté des sciences de Sfax. Thèse,113p.
40. Gopal, J., K. Iwama, 2007. *In vitro* screening of potato against water stress mediated through sorbitol and polyethylene glycol. *Plant Cell Report,* 26, 693-700.
41. Bayoumi TY, Manal HE, Metwali EM, 2008. Application of physiological and biochemical indices as a screening technique for drought tolerance in wheat genotypes. *African Journal of Biotechnology*. 7:2341-2352.
42. Wani SH, Sofi PA, Gosal SS, Singh NB, 2010. *In vitro* screening of rice (*Oryza sativa* L.) callus for drought tolerance. *Communications in Biometry and Crop Science*. 5:108-115.
43. water stress in barley: a QTL study. *New Phytol.* 37, *99-107.*
44. Tschaplinski T. J., Gebre G. M., Dahl J. E., Roberts G. T. et G. A. Tuskan, 1995. Growth and solute adjustment of calli of *Populus* clones cultured on nutrient medium containing polyethylene glycol. *Can. J. For. Res.,* 1425-1433.
45. Afm Van Hees. 1997. Growth and morphology of pedoncule oak (*Quercus robur* L) and beek (*Fagus sylvatica*) seedlings in relation to shading and drought. *Ann. Sci. For*. 54, 9-18.
46. Throught M. C. T. et Drew M. C. 1980. The development of water logging damage in weat seedlings (*Triticum aestivum* L.). Shoot and root grouth relation to the changes in concentrations of dissolved gases and solutes in the soil solution, *Plant Soil*m, 77-94.
47. El Jaafari S. 2000. Durum wheat breeding for abiotic stresses resistance: Defining physiological traits and criteria. *Option méditerranéenne*. 40:251-256.
48. Slama I, Messedi D, Ghnaya T, Savouŕe A, Abdelly C (2006) Effects of water-deficit on growth and proline metabolism in *Sesuvium portulacastrum*. Environ Exp Bot 56:231–238.
49. Mohsenzadeh S., Malboobi M. A., Razavi K., Farrahi-Aschtiani S. 2006. Physiological and molecular responses of Aeoluropus Lagopides (Poaceae) to water deficit. *Env. Exp. Bot*. 56:314-322.
50. Matin M. A., Jarvis H. B and Hayden F. 1989. Leaf water potential, relative water content, and diffusive resistance in Barley. *Agron. J.,* 81:100-105.
51. Diaz-Perez J. C., Shekel K. A and Sutter E. G., 1995. Relative water content and water potential of tissues cultured apple shoots under water deficit. *Journal of Experimental Botany*. 46:111-118.
52. Tahri E. H., Belabed A. et Sadki. 1998. Effet d'un stress osmotique sur l'accumulation de praline, de chlorophylles et des ARNm codant pour la glutamine synthétase chez trios variétés de blé dur (*Tritucum durum*). *Bull. Inst. Sci.,* 21,81-87.
53. Scheirs et De Bruyn. 2005. Plant mediated effects of drought stress on host preference and performance of a grass miner. *OIKOS* 108:371-385.
54. Nabors M. W., Gibbs S. E., Bernstein C. S. and Meis M. E., 1980. NaCl-tolerant tobacco plants from cultured cells. Z. *Pflanzenphysiol.* 97:13-17.
55. Bouharmont J. 1991. Utilisation de la variation somaclonale et de la sélection *in vitro* à l'amélioration du riz. Ed. AUPELF-UREF. John Libbey Eurotext. Paris.1-8.
56. Hsissou D. and Bouharmont J. 1994. *In vitro* selection and characterization of drought tolerant plants of durum wheat (Triticum durum Desf.). *Agronomie*. 2:65-70.
57. Kavirishor P. B. K and Reddy M. G., 1985. Resistance of rice callus tissues to sodium chloride and poly ethylene glycol. *Curr. Sci.,* 54:1129-1131.
58. Adkins S. A., Kunanuvatchaidach R. K. and Godwin I.D. 1995. Somaclonal variation in rice drought tolerance and other agronomic characters. *Austral. J. Bot*. 43:201-209.
59. Smith RH, Bhaskaran S, Miller FR, 1985. Screening for drought tolerance in Sorghum using cell culture. *In vitro Cell. Dev. Biol.* 21,541-545.
60. Milache B. E., 1970. Carbowax-6000 compared with mannitol suppressant of cucumber hypocotyl elongation. *Plant Physiol*. 45:507-509.