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Callus Formation and Production of Secondary Metabolites by Seedling Explants of *Chenopodium quinoa*

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QUINOA (*Chenopodium quinoa* Willd) which is considered a pseudocereal or pseudograin, has been originated from the Andean region in South America, and is belonging to family Amaranthaceae. The highest percentage germination of seeds was achieved in MS medium with full strength in the full strength MS medium (100%). Best callus production from seedling explants was obtained on MS medium supplemented with 2mg/L 2, 4-D+0.05mg/L Kin. Callus was also obtained when MS medium was fortified with 1mg/L NAA+0.5mg/L BA, 1mg/L 2, 4-D+0.5mg/L BA and 3mg/L PCIB. However, the percent culture response on these concentrations was lower. The lowest total amount of callus was found to be on MS medium containing 1mg/L PCIB. Callus of explants were grown on MS media with 2mg/L 2,4-D+0.05 mg/L Kin gave the significant highest value (22µg/g fresh wt) of tocopherols content, followed by seedling on half strength MS medium (15.6). While the lowest value (2.1) was observed with Callus obtained from seedling planted on MS medium contained 0.5mg L^{-1} NAA+0.05 mg L^{-1} BA.

Protein extraction and enzymatic assay protein extraction and measurement of tyrosine aminotransferase (TAT) activity were performed. Enzyme activity in leaves was twofold higher than that in seeds. In callus cultures, activity was about onefold lower than in leaf extracts.

Keywords: *Chenopodium quinoa* (L.), NAA, PCIB, Callus formation, 2, 4-D and tocopherols.

Introduction

Chenopodium quinoa is an herbaceous dicotyledonous crop belonging to the C3 group of plants. It is a member of the Amaranthaceae family and is called a pseudo-grain plant. Pseudocereals are plants that produce seeds and fruits that are similar to grains and used in virtually the same ways. High in protein, nutrients, and vitamins, pseudocereals are most commonly gluten free and considered whole grains, despite not being a true grain (Jacobsen et al., 2003). Quinoa appears as an important crop with the potential to contribute to food safety worldwide and is considered to be an optimal food source for astronauts.

Quinoa has recently become particularly popular because it is gluten-free. Moreover, it has a high nutritional value with an average of 14.8% protein and an exceptional balance of oil, protein and starch, (Wright et al., 2002 and Tang et al., 2015). Quinoa seedlings are a very nutritious being rich in amino acids (lysine), unsaturated fatty acids (linolenic acid, linoleic acid), minerals as co-factors in antioxidant enzymes (calcium, magnesium, iron, copper and zinc), tocopherols (vitamin E), saponins and phenolic compounds with antioxidant power (Rengasamy, 2006 and Vega-G´alvez et al., 2010). Moreover, quinoa exhibits not only an unusually high salt tolerance but it also can grow under extreme drought stress (Jacobsen et al., 2003). The main problems with quinoa culture is its susceptibility to many viral diseases which seeds of infected plants carry the virus transferred to next generation. One way to produce virus-free plants is the *in vitro* propagation. Low-frequency chimeric somatic embryos and a high number of regenerates with a limited level of somaclonal variation are more attractive than organogenesis as a plant regeneration system (Henry, 1998). The use of *in vitro* culture techniques for study and select of improved cell lines are also very useful tools because they offer a rapid selection on a mass scale. Plant cell, organ, and tissue culture have widely used as a tool for conventional crops production (Collonnier et al., 2001; Basu et al., 2002; Pauk et al., 2002; Borsani et al., 2003; Cherian & Reddy, 2003; Zair et al., 2003 and Compton et al., 2004)

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and halophytes (Cherian & Reddy, 2002, 2003).

Tocopherols, collectively known as vitamin E, are lipophilic antioxidants, essential nutritional components for mammals and exclusively synthesized by photosynthetic organisms. Of the four forms (α, β, γ and δ), α-tocopherol is the most important vitamin E form present in green plant tissues and has the highest vitamin E activity. Synthetic α-tocopherol, which is a racemic mixture of eight different stereoisomers, is always less effective than α-tocopherol the natural form (R, R, R). This increases the interest in obtaining this molecule from natural sources, such as plant cell cultures (Caretto et al., 2010).

Tyrosine aminotransferase (TAT) catalyses the reversible reaction of tyrosine + 2-oxoglutarate ↔ 4-hydroxyphenylpyruvate + glutamate. In bacteria, tyrosine is produced from 4-hydroxyphenylpyruvate by TAT, but in plants it has been described that the enzyme works in the other direction (Buchanan et al., 2000), conversion of tyrosine to 4-hydroxyphenylpyruvate, which is the substrate for pathways producing plastoquinone, tocopherols, rosmaric acid and benzylisoquinoline alkaloids (Lee & Facchini, 2011).

Here we examine a simple strategy for somatic embryogenesis using hypototyl and cotyledonal plants from *Chenopodium* seedlings that can contribute to further progress in this area.

Materials and Methods

Plant material, sterilization and preparation of explants

Seeds of quinoa (*Chenopodium quinoa* Willd.) were obtained from Agricultural Research Centre Giza, Egypt. The experimental design was in randomized complete block with four replications.

Seeds sterilization and germination

The seeds were carefully washed with detergent (0.003% (w/v) of Tween 20) and rinsed with tap water for 10min. They were sterilized with sodium commercial hypochloride 60% for 20min. The seeds were carefully washed with 3 time's sterile distilled water for 15min.

Then, the sterilized seeds were plated on either full strength or half strength (Murashige & Skoog, 1962) MS medium supplemented by 0.2g gelrite and 30g sucrose.

The cultures were kept in a growth chamber at 26±1˚C, and a photoperiod of 16h lights (30μE. $m^{-2}sec^{-1}$,, Philips TL 33 light) and 8h dark.

Callus induction

The seeds were germinated within 10 days and these *in vitro* germinated seeds were used as a source of explants for further experiments. Explants with 0.5–1.0cm length, from hypocotyl and (0.5cm^2) cotyledons of 10 days-old seedlings were cultured on a basal MS medium with different concentrations of plant growth regulators (Duchefa biochemistry, Netherlands) for callus induction (Table 1). All cultures were incubated at 25±2°C in the dark. To give a dark condition, impervious light plastic was used. After 4 weeks, calli were produced and transferred to light for 2 weeks and weighed.

TABLE 1. **Different hormone treatments for callus establishment of *Chenopodium quinoa* used to test extracts for tocopherol content.**

No	Medium Type
M1	MS medium + 0.5mg L^{-1} NAA*+0.05mg L^{-1} BA
M2	MS medium + 1mg L^{-1} NAA*
M3	MS medium + 1mg L^{-1} NAA+0.5mg L^{-1} KIN**
M4	MS medium + 1mg L^{-1} NAA+0.5mg L^{-1} BA***
M5	MS medium + 1mg L^{-1} NAA +0.05mg L^{-1} BA
M6	MS medium + 2mg L^{-1} 2,4-D****+ 0.5mg L^{-1} BA
M7	MS medium +2mg L^{-1} 2,4-D+0.05mg L^{-1} KIN
M8	MS medium + 3mg L^{-1} PCIB*****
M9	Half MS
M10	Full MS
M11	seed

* Naphthalene acetic acid
** Kinetin
*** Benzyle adenine
**** 2, 4-Dichloroacetic acid
***** p-Chlorophenoxyisobutyric acid

Extraction of tocopherols

Extraction of tocopherols from seeds, leaves of seedling and callus cultures of *C. quinoa* (Table 1) was carried out taking care to protect the extracts from light and oxidizing conditions. A total of 5g freeze-dried material or dry seeds were ground to a powder and stirred with 100ml methanol for 3h in the dark at 25°C, then sonicated in a Branson ultrasound bath for 40min. After filtration and centrifugation at 3000*g* for 20min, the supernatant was dried in a rotary evaporator and weighed. The dried extract was resuspended in 100ml *n*-hexane,

sonicated again for 40min, and then centrifuged at 3000*g* for 20min. The supernatant was dried and weighed and stored at 20°C until further use.

HPLC analysis of tocopherols

Extraction of tocopherols from seeds, leaves of seedling and callus cultures of *C. quinoa* (Table 1) was carried out in dark to protect the operations from light and oxidizing conditions. A total of 5g freeze dried material or dry seeds were ground to a powder and stirred with 100ml methanol for 3h in the dark at 25°C, then sonicated in a Branson ultrasound bath for 40min. After filtration and centrifugation at 3000*g* for 20min, the supernatant was dried in a rotary evaporator and weighed. The dried extract was resuspended in 100ml *n*-hexane, sonicated again for 40min, and then centrifuged at 3000*g* for 20min; the supernatant was dried and weighed, and stored at 20°C until further use.

HPLC analysis of tocopherols

Analyses were performed essentially as described by Bruni et al. (2004); accurately a proper quantity of investigation sample weighed into a 20ml glass stoppered-Erlenmeyer flask. 1 ml of the solution composed of approximately 25mg of 2, 2, 5, 7, 8-pentametyl-6-hydroxychroman or 2-metyl-2-phytyl-6-hydroxychroman into a beaker and dissolved in 100ml n-hexane added to the flask using a whole pipette, and dilute with n-hexane to about 10ml. Then, the solution was filtered through a membrane filter of a 0.45µm pore-size. the filtrate utilized as test solution for HPLC analysis.

Protein extraction and TAT activity

Protein extraction and enzymatic tests Protein extraction and measurement of tyrosine aminotransferase (TAT) activity were performed as described previously in Lopukhina et al. (2001). Total RNA from tha plant samples sprayed with 5uM coronatine and incubated for 2h was extracted as described by Barkan (1989). Poly (A^+) RNA was isolated using Oligotex mRNA Kit (Oliagen, Hilden, Germany).

Enzyme activity was assayed using 100µg of total protein, as described by Lopukhina et al. (2001). Protein content was determined according to Lowry et al. (1951). Five g fresh plants were homogenized in 2 volumes of ice-cold buffer, containing 100mM potassium phosphate buffer (pH 7.5) and 0.1mM EDTA. The supernatant was made after filtration 0.1% (v/v) with tergitol and an incubation period on ice followed for 15min. The supernatant after centrifugation was utilized as crude enzyme extract.

The experiments were repeated twice, with three replicate measurements. Data were analyzed using the one-way ANOVA test with a significance threshold of $P \leq 0.05$.

Statistical analysis

Thirty explants were cultured per treatment. Each treatment consisted of 40-50 polypropylene jars (5cm high) with 5 explants in each jar. In the table, we show percentages and means ± SE. The Student *t*-test and the $\chi 2$-test were used to evaluate the significance of differences with averages and percentages respectively. The tests were carried out at least twice and comparable results were achieved. The results were statistically analyzed by a factor analysis of variance, in fully randomized design according to the procedure at Snedecor & Cochran (1981) and means were compared by multiple range tests.

Results and Discussion

The first experiment was set up to find the most appropriate MS strength. The results showed that the interaction between MS strength and standard photoperiodicity had a significant effect on seed germination and that the highest percentage of germination (100%) was achieved at full strength MS medium (Fig.1). By an increase of the concentration to full strength MS, the germination percentage was significantly increased. This is in agreement with Eisa et al. (2005) who have reported that acceptable seed germination of *C. quinoa* was observed in full strength of MS medium. The germination responses of halophytic seeds counting salinity, temperature, light and life form were determined by several factors (William et al., 1998)

The second experiment was set up to find out an appropriate combination of plant growth regulators for callus induction. Cotyledon segments gave the best callus initiation and, among the different media tested, maximum callus formation was achieved with 2.0mg L^{-1} 2,4-D and 0.05mg L^{-1} KIN (Table 2). Callus induction was also quite high with hypocotyl explants (95%) at the same 2,4-D and KIN concentration. Eisa et al. (2005) reported that callus formation was achieved from hypocotyl segments of *C. quinoa* but not from root and cotyledon explants. In the current study,

hypocotyls explants were used for callus formation. The results showed that maximum callus weight (1.22g) and percent of callus induction (95%) was obtained on MS medium supplement with 2.0mg L^{-1} 2,4-D and 0.05mg L^{-1} KIN (Table 2 and Fig. 2 b). This is not in agreement with Eisa et al. (2005) who have reported that acceptable callus formation from hypocotyls explants of *C. quinoa* was observed in medium supplemented with 0.1 to 0.5mg L^{-1} 2,4-D.

We also examined PCIB which is a genuine antiauxin. Interestingly, this compound resulted in lowest callus formation.

The use of plant growth regulators is of fundamental importance for controlling the organogenic response of each plant tissue/organ under in vitro conditions (Che et al., 2002 and Sugiyama & Imamura, 2006). Studies have shown that 2,4-D is one of the most effective auxins for induction and growth of callus (Lee et al., 2004 and Burbulis et al., 2007). Hypocotyls of young seedlings are often used for in vitro regeneration of different plants (Campbell & Durzan, 1975; Gamborg & Shyluk, 1976; Gunay & Rao, 1978; Kamat & Rao, 1978; Fari & Czako, 1981; Kameya & Widholm, 1981 and Arrilaga et al., 1986). However, Al-Khayri et al. (1992) achieved callus formation from mature seed explant of *Spinacia oleracea* L. on medium supplemented with 0.75mg L^{-1} 2,4-D along with 2mg L^{-1} KIN. Bhojwani & Dantu (2013) reported that the texture and color of the callus are markers for regeneration. Callus size is also an important factor in survival of callus. Survival rates of small callus compared to large callus are less, which may be due to specific cell density (Hesami & Daneshvar, 2016), so that they can independently continue to grow after separation from the explant. So in this experiment, bulk of callus from MS medium supplement with 2mg L^{-1} 2,4- D along with 0.05mg L^{-1} KIN.

Tocopherols content

Phenolics or polyphenols have received considerable attention because of their physiological characteristics, including antioxidant, antimutagenic and antitumour activities (Othman et al., 2007).

In *C. quinoa* cultures, the production of tocopherols was not influenced by the exogenous hormone supply, or by the presence/absence of chloroplasts

The total content of tocopherols ranged from 2.1 to 22µg g^{-1} FW (highest in M7) (Fig. 3) in different callus parts, seed and seedlings samples, which was similar to that in many fruits (1.1–84 µg g^{-1} FW), vegetables (1.0–30µg g^{-1} FW), legumes (4.8–16.7µg g^{-1} FW) and cereals (17–60µg g^{-1} FW) reported earlier by Caretto et al. (2010). The most abundant tocopherol in *C. quinoa* leaves was α-tocopherol followed by γ- and δ-tocopherol. α-Tocopherol is reported to have greater vitamin E activity and occurs in leaf tissues of various plant species (Carvalho et al., 2013). The content of α-tocopherol in *C. quinoa* leaves (101.1µg g^{-1} FW) was higher than the value reported in *Amaranthus caudatus* (11.3µg g^{-1} FW), *Arabidopsis* (10µg g^{-1} FW), *Chenopodium quinoa* (1.98µg g^{-1} FW), sunflower (14µg g^{-1} FW) and tobacco (43µg g^{-1} FW) cell cultures (Gala et al., 2005; Antognoni et al., 2008 and Harish et al., 2013) while lower than the value reported in *Carthamus tinctorius* (167.7µg g^{-1} FW) and *Vitis vinifera* (261.5µg g^{-1} FW) cell cultures (Chavan et al., 2011 and Cetin, 2014).

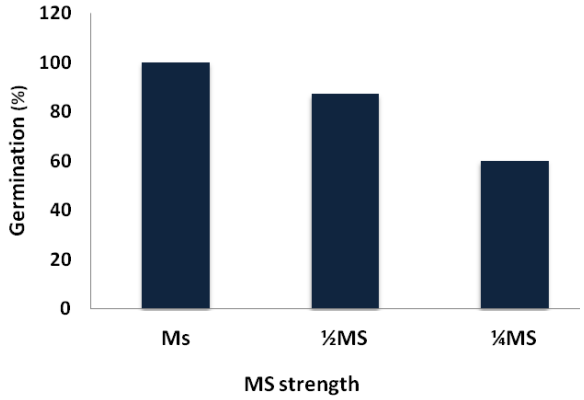

Fig. 1. Effect of different strength of MS medium on percentage f seed germination of *Chenopodium quinoa*.

TABLE 2. Effect of different concentrations of growth regulators on callus induction in hypocotyls and cotyledon explants of *Chenopodium quinoa* and different letters within columns indicate significant differences (P< 0.05).

	Callus formation (%)	FW callus (g)	DWcallus (g)
1mg L^{-1} NAA	53±3.7d	1.5±0.04c	0.15±0.02c
0.5mg L^{-1} NAA+0.05mg L^{-1} BA	47±6.3e	1.0±0.14d	0.1±0.042d
1mg L^{-1} NAA+0. 5mg L^{-1} K	65±4.5c	1.3±0.13d	0.15±0.056d
1mg L^{-1} NAA+0.5mg L^{-1} BA	46± 6.6e	1.3±0.102d	0.13±0.12d
1mg L^{-1} NAA+0.05mg L^{-1} BA	55± 4.1d	1.6±0.021c	0.16±0.04c
3mg L^{-1} NAA	67±6.5c	2.3±0.09b	0.23±0.03b
1mg L^{-1} 2,4D	71±5.1b	2.4±0.044b	0.22±0.02b
2mg L^{-1} 2,4D+0.05 mg·l^{-1} KIN	95±3.1a	3.6±0.18a	0.36±0.12a
1mg L^{-1} 2,4D+0.5mg L^{-1} BA	88±5.2a	3.1±0.13a	0,32±0.033a
2mg L^{-1} 2,4D+0.05mg L^{-1} BA	77±6.3b	2.6±0.166b	0.25±0.056b
1mg L^{-1} 2,4D+0.5mg L^{-1} KIN	90±7.1a	2.8±0.154b	0.29±0.012b
1mg L^{-1} PCIB	69±7.5c	2.0±0.132b	0.2±0.055b
3mg L^{-1} PCIB	44±3.4e	1.9±0.1c	0.19±0.17c

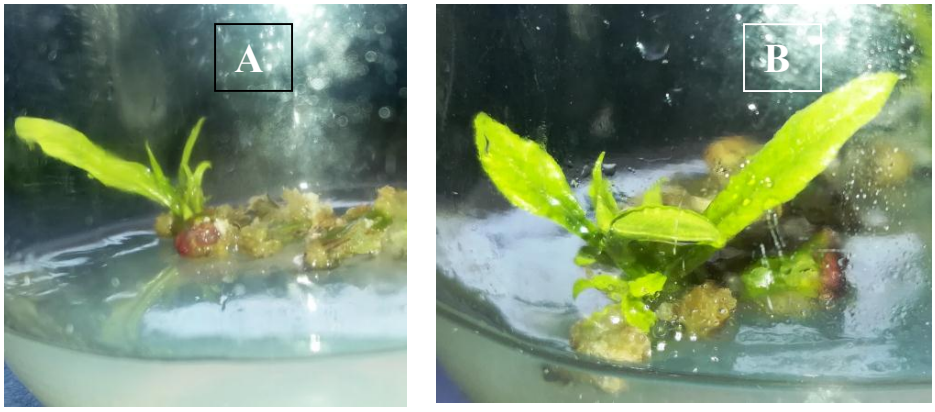

Fig. 2. Effect of different growth regulators with different concentrations on callus induction with indirect regeneration in hypocotyls explants of *Chenopodium quinoa*, (A) MS medium containing 1mg L^{-1} NAA +0.05 mg L^{-1} BA and (B) MS medium containing 1mg L^{-1} 2,4D+0. 5mg L^{-1} KIN.

An inverse relationship between growth rate and secondary metabolite production in plant cell and tissue cultures has often been reported (Hagendoorn et al., 1997 and Collin, 2001). On the other hand, they have a well-demonstrated antioxidant function and are known to increase in plants exposed to adverse environmental conditions that induce oxidative stress (Delong & Steffen, 1997 and Havaux et al., 2005), thus behaving as secondary metabolites.

In callus cultures of *C. quinoa*, α-tocopherol levels were about ten to 40 times lower than in leaves, irrespective of the culture medium used. This is in line with the general assumption that undifferentiated *in vitro* cultures, except for a few well-known exceptions (e.g., taxanes, Yukimune et al., 1996), often lose the capacity to produce the secondary metabolites normally synthesized in plant. In some cases, this is due to a lack of specialized cells or secretory structures (St-Pierre et al., 1999 and Pasqua et al., 2003). Indeed, the existence of a relationship between differentiation/ organization and secondary product formation *in vitro* has been well documented (Robins et al., 1991 and Biondi et al., 2002).

Tyrosine aminotransferase (TAT) activity in callus cultures

TAT activity was measured in protein extracts from seeds, leaves and callus cultures of *C. quinoa*. Enzyme activity in leaves was twofold higher than that in seeds (Fig. 4). In callus cultures, activity was about onefold lower than in leaf extracts, independent of the medium used.

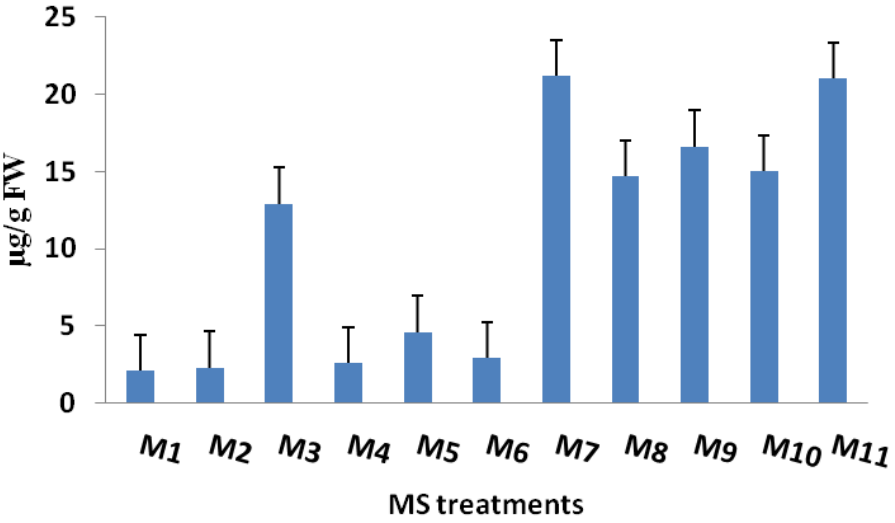

Fig. 3. Tocopherol levels in 7-week-old explants Influenced by various growth regulators added to MS medium on callus fresh weights obtained from *in vitro* hypocotyl of *Chenopodium quinoa* [Data± standard error].

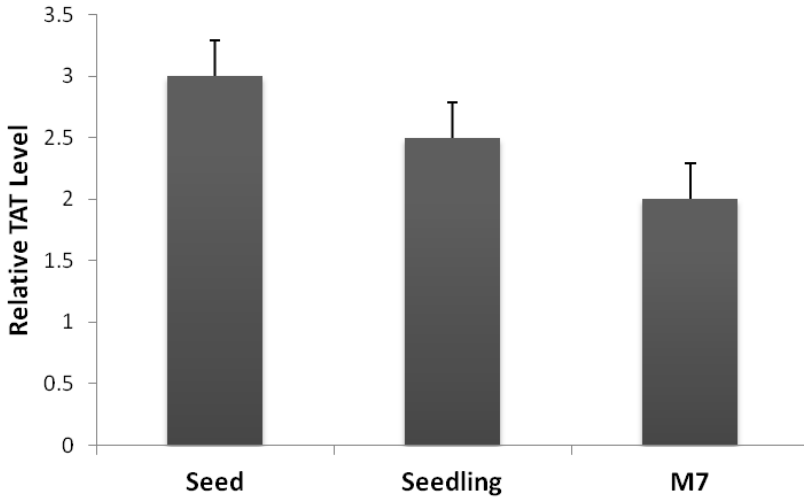

Fig. 4. Relative TAT level in different Chenopodium organs and callus at treatment 1mg L^{-1} 2,4D+0. 5mg L^{-1} KIN [Values represent means ± SE of triplicate experiments].

Lopukhina et al. (2001) showed that the tyrosine aminotransferase annotated by locus tag At4g23600 from *A. thaliana* was regulated by coronatine. TAT was found to implicate in tocopherol anabolism in plants (Lopukhina et al 2001 and Hollander-Czytko et al., 2005). Additionally, the enzyme was also shown to be involved in the synthesis of rosmarinic acid in whole plants and in hairy root cell cultures (Xiao et al., 2009).

Conclusion

Conditions for initiation of callus in hypototyl and cotyledonal explants from Chenopodium seedlings were optimized in this study. Callogenesis showed range of responses depending on medium formulation, growth regulators combinations and concentrations. Various combinations and numerous medicinal properties of its extract and essential oils demand further and more studies about the other useful and unknown properties of this multipurpose plant.

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دراسة تكوين الكالس وانتاج المركبات الثانوية من البادرات لنبات الكينوى

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تعتبر الكينوا (*Chenopodium quinoa* Willd) من البذور الكاذبة، وقد نشأ من منطقة الأنديز في أمريكا الجنوبية، وينتمي إلى عائلة Amaranthaceae. تم تحقيق أعلى نسبة إنبات بذور في وسط MS مع القوة الكاملة في المتوسط MS الكامل (100%). تم الحصول على أفضل إنتاج الكالس من مستخلصات الشتلات على وسط ال MS مدعوم بـ 122/mg، 4-D + 0.05 mg/1Kin. تم الحصول على الكالس أيضا عندما تم تحصين MS المتوسطة مع 1 1BA / 0.5mg + 1NAA/mg، 12 / 1BA / 0.5 mg + 4-D و 3 PCIB l / mg. ومع ذلك، كانت استجابة الثقافة المئوية على هذه التركيزات أقل. تم العثور على أقل كمية إجمالية من الكالس على وسط MS تحتوي على 1 ملغ / لتر PCIB. نمت الكالس من explants على وسائل الإعلام MS مع 0.052 + 4-D، 12 / mg ملغ / Kin1 أعطى أعلى قيمة (22 ميكروغرام / غرام من وزن جديد) من توكوفيرولس، تليها الشتلات على متوسط القوة المتوسطة (15.6). في حين لوحظ أدنى قيمة (2.1) مع كالوس التي تم الحصول عليها من الشتلات المزروعة على المتوسطة MS تحتوي على 0.5 ملغ ل0.05 + 1 NAA - ملغ • ل 1- BA.

تم إجراء استخراج البروتين والإنزيمي مقايسة استخراج البروتين وقياس نشاط التيروزين aminotransferase (TAT). كان نشاط الإنزيم في الأوراق ذات شقين أعلى من ذلك في البذور. في الثقافات الكالس، كان النشاط حول عنيف أقل من مستخلصات الأوراق.