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BIOCHEMICAL CHARACTERISTICS AND NUTRIENT CONTENT OF THE CALLUS OF SUNFLOWER INBRED LINES

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SUMMARY

Mineral nutrition is a factor affecting *in vitro* regeneration. Various requirements for individual mineral elements between plant species and also between genotypes of the same species have led to the differentiation and definition of the significance of the genetic background in determining callus biochemical characteristics and a confirmation whether different requirements for nutrients leave a possibility of achieving better and more efficient regeneration with a specific modification of substratum composition. The practical aspect of the obtained results lays in the fact that the sunflower species are characterized by low regeneration ability. It was shown that the dynamics of uptake and accumulation of mineral elements into callus cells is a genotype dependent trait being more or less evident depending upon element. Total nitrogen content was between 4 and 5%. The sunflower genotype PH-BC_2-101A showed the highest accumulation of nitrogen, phosphorus and, to some extent, calcium. Consequently, a high accumulation of total dry matter without pigment synthesis was recorded. A high nitrogen accumulation in the cited genotype resulting in the highest nitrate reductase activity a very high accumulation of soluble proteins (enzymes).

Key words: **sunflower inbreds, calluses, mineral nutrition, soluble proteins, pigments, nutrients uptake**

INTRODUCTION

In defining parameters limiting plant growth and production, an important place belongs to monitoring the rate and degree of *in vitro* propagation from different tissues (callus culture, protoplast culture, shoot induction on callus, *etc.*). Improvement of sunflower characteristics by tissue culture methods is frequently

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accompanied by difficulties in plant regeneration. This limits the use of those methods in a reproducible and efficient way (Vasić *et al.*, 2001). A primary factor affecting *in vitro* regeneration is mineral nutrition. Plant tissue culture requires the optimization of growth media. The production of biomass and secondary metabolites may be manipulated by changing nutrient composition (Morard and Henry, 1998). However, few literature data are available on a precise definition of parameters affecting actual requirements, uptake, and redistribution of the individual macro- and microelements in *in vitro* culture.

A precise definition of nutrient requirements is an important task, in particular taking into consideration the fact that, frequently, favorable ecological conditions and optimum combination of growth-regulating substances may not produce viable explants with a satisfactory growth rate. The genetic background impact upon callus metabolism and consequently upon metabolite and mineral element concentrations is noteworthy and the concentration of elements of mineral nutrition may be highly genotype-, *i.e.*, explant-type-dependent (Mezei *et al.*, 1995). For optimization of *in vitro* growth conditions to enhance the regeneration, favorable concentration and ratio of the individual nutrients required for specific genotypes should be defined (Ravishankar and Grewal, 1991). To define a model of optimum mineral nutrition, it is necessary to analyze the chemical composition of genotypes and their biochemical characteristics; those characteristics are determined by the medium composition due to a heterotrophic callus character (Bozkov, 1995; Ketchum, 1995). By applying a systematic approach in surveying the growth rate and nutrient concentration (in nutrient solution and plant tissue), distinct reasons for nutrient deficiency may be distinguished and consequently solutions for plant growth problems generated either by nutrient deficiency or their disturbed ratio could be found (Leifert, 1995).

Also, *in vitro* plant culture where explants of different origin are used for callus induction (different genotypes, different cells of the same genotype) may ensure some degree of somaclonal variation, which is of great practical value in breeding and development of genotypes with desirable traits (Zhong *et al.*, 1993).

The aim of this paper was to distinguish and define the importance of the genetic background in determining the biochemical characteristics of callus and to establish whether different requirements for nutrients leave possibilities of accomplishing better and more efficient regeneration using a specific modification of substratum composition. Such a survey has a practical value taking into consideration that cultivated sunflower is characterized by low regeneration ability. The above facts open up the possibility of developing novel technologies of sunflower growing by conducting selection in a desirable direction.

MATERIAL AND METHODS

Experiments were conducted on the callus tissue of six sunflower inbred lines of different origin and characteristics: PH-BC_1-162A, PH-BC_2-91A, PH-BC_2-101A, CMS-3, CMS-8931-3-4-A and Ha-48A. The plant material was taken from the Novi Sad sunflower breeding program. Each inbred line was represented by 10 plants,

which were donors of initial explants. Calluses induced from hypocotyls were grown according to the protocol of Paterson and Everett (1985). Hypocotyl explants from eight-day old seedlings were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 1 mg l^{-1} BAP, 1mg l^{-1} BAP and 0.1 mg l^{-1} GA_3. They were grown two weeks in the dark and two weeks in the light (photoperiod 16 light : 8 dark), at 25°C. After that period of time, calluses were rinsed with distilled water and prepared for analyses. Extraction of soluble proteins from fresh sunflower calluses was made by 0.1 mol dm^{-3} phosphate buffer, pH 7, and protein content was determined after Lowry (1951), using bovine serum as standard. Nitrate reductase activity was determined in callus tissue by an *in vivo* method, in phosphate buffer, pH 7.4 (Hageman and Reed, 1980). Chlorophyll (Chl *a* and Chl *b*) and carotenoid (Car) contents were determined spectrophotometrically after extraction and filtration in absolute acetone; equations of Lichtenthaler and Wellburn (1983) were used for quantifications.

Mass of fresh and dry matter (after drying at 105°C) of each callus was measured, and dry matter content was expressed in percentage on fresh matter basis.

Prior to chemical analysis, plant material was dried and milled. Total N concentration in callus dry matter was estimated by standard microkjeldahl method (Nelson and Sommers, 1973). The concentrations of P, K, Ca, Mg, Fe, Mn, Cu and Ni were determined after dry ashing at 450°C and treatment with HCl. Then phosphorus was assayed spectrophotometrically by ammonium-vanadate-molybdate method, potassium by using a flame photometer, and other elements by atomic absorption spectrophotometer.

Data analysis was done using multiple interval test (Duncan), and testing was done for the level of significance of p=0.05.

RESULTS AND DISCUSSION

Significant discrepancies were recorded between the genotypes in the contents of the surveyed macro - and micronutrients (Table 1).

Table 1: Concentration of some macro and micronutrients in calluses of six sunflower inbreds

Genotype	Macronutrient (g ·100g^{-1} dry matter)					Micronutrient (mg ·kg^{-1} dry matter)			
	N	P	K	Ca	Mg	Fe	Mn	Cu	Ni
PH-BC_1-162A	4786 b	281 c	5742 a	85 cd	258 a	299 e	120 c	29 a	9.23bc
PH-BC_2-91A	4323 c	360 b	6011 a	81 d	229 b	368 d	110 d	27 a	7.61 d
PH-BC_2-101A	5310 a	473 a	5237 b	100 ab	215 c	515 b	125 b	20 b	12.07 a
CMS-3	4866 b	285 c	5816 a	108 a	254 a	483 c	120 c	16 c	8.57cd
CMS-8931-3-4-A	4757 b	299 c	5155 b	95 bc	209 c	482 c	126 b	19 bc	9.82 b
Ha-48a	4145 c	192 d	4538 c	105 ab	214 c	572 a	136 a	27 a	11.71 a

Values with the same letter within the same column do not differ significantly at p=0.05

These obtained differences point to different requirements for nutrients among sunflower inbred lines therefore showing that variation of concentration and ratio

of nutrients in a medium may have a great impact on enhanced regeneration rate. The genotype PH-BC_2-101A showed the highest nitrogen and phosphorus accumulations and also a high accumulation of calcium. This resulted in a high accumulation of total dry matter without pigment synthesis due to the lowest concentration of total photosynthetic pigments. Low concentration of total photosynthetic pigments was also found in the genotype PH-BC2-91A (Table 2).

Table 2: Some biochemical parameters in calluses of six sunflower inbreds

Genotype	Soluble proteins (mg g^{-1} fresh mass)	Nitrate reductase activity (μmol NO_2 $g^{-1}h^{-1}$)	Dry matter (%)	Chl a (mg g^{-1} dry mass)	Chl b (mg g^{-1} dry mass)	Carotenoids (mg g^{-1} dry mass)
PH-BC_1-162A	16.53 a	4.64 ab	5.29 b	1.21 a	0.55 a	0.47 a
PH-BC_2-91A	8.01 c	3.43 cd	7.66 a	0.24 d	0.02 e	0.14 c
PH-BC_2-101A	13.99 b	5.43 a	7.85 a	0.28 d	0.24 c	0.14 c
CMS-3	16.02 ab	3.13 d	8.56 a	0.51 c	0.21 c	0.23 b
CMS-8931-3-4-A	17.75 a	3.88 bcd	4.28 b	0.98 b	0.35 b	0.44 a
Ha-48a	7.83 c	4.17 bc	3.84 b	0.41 cd	0.10 d	0.16 c

Values with the same letter within the same column do not differ significantly at p=0.05

Intensity and dynamics of callus growth rate are influenced by nitrogen content and form (Elkonin and Pakhomova, 2000). Ammonium and nitrate uptakes and their metabolisms in cells significantly determine the biochemical characteristics of the callus (Curtis and Smarelli, 1987; Feng and Ouyang, 1988). The concentration of total nitrogen in the surveyed calli resembled that recorded most frequently in leaves of intact plants (Sfredo *et al.*, 1985; Sarić *et al.*, 1991), whereas the percentage of soluble proteins within total proteins was genotype-dependent (Table 2). By comparing the data on the content of soluble proteins in leaves (Kastori *et al.*, 1992) and sunflower calli, it was concluded that the calli of the genotypes PH-BC_1-162A, PH-BC_2-101A, CMS-3, and CMS-8931-3-4-A showed similar contents of soluble proteins when compared with intact leaves. The percentage of soluble proteins in total proteins points to the physiological, *i.e.*, enzyme activity in callus cells. A significantly lower percentage of soluble proteins (enzymes) in the calli of the lines PH-BC_2-91A and Ha-48A when compared with the intact sunflower leaves, showed an elevated nitrate, *i.e.* ammonium ion uptake and their accumulation in cells in the earliest stages of their development. In later stages, the intensive divisions and differentiation during callus formation led to significant incorporation of accumulated nonprotein nitrogen into polypeptides, *i.e.* enzymes. One of the most important enzymes taking part in nitrogen fixation and ammonium incorporation into proteins is glutamine synthetase (Magalhaes and Huber, 1991).

By comparing the above data with the content of soluble proteins in sugarbeet calli of similar age (unpublished results) it was concluded that the percentage of soluble proteins was significantly higher in sunflower calli than in the calli of different sugarbeet genotypes.

Montoro *et al*. (1995) also reported on such a dynamics of nitrogen uptake and metabolism in calli. As callus growth parameters may be expressed through nitrate uptake and accumulation, increase of fresh and dry mass and protein accumulation, a suitable indicator of these parameters is also the activity of nitrate reductase (Roberts *et al*., 1996). Relatively low values of nitrate reductase activity, despite a high nitrogen concentration, show that callus did not achieve maximum intensity rate of nitrogen metabolism in the surveyed stage of development (Table 2). Genotype specificity of the surveyed parameters of the nitrogen metabolism was evident. Emphasis should be placed on the genotype PH-BC_2-101A which showed the highest accumulation of this macronutrient, resulting in the highest activity of nitrate reductase and a very high accumulation of the soluble proteins. Consequently, calli of this genotype could be distinguished as the most active metabolically, as they were also characterized by the highest percentage of dry matter.

The genotype PH-BC_2-101A also had very high accumulations of the other macronutrients, except Mg (Table 1). Mg concentration in callus cells, by activating the plasma membrane H^+-ATPase, influences cytosolic pH, *i.e.* pH gradient across membranes (Costa and Meis, 1996). High Mg accumulation increases cellular pH and decreases pH gradient across cellular membrane resulting in a decrease in nitrogen uptake, particularly of the ammonium form (Schmitz and Lorz, 1990). This antagonism between N and Mg was particularly evident in the genotype PH-BC_2-101A.

Genotype specificity was also observed in the concentration of the surveyed macronutrients. The genotype Ha-48A, characterized by the lowest concentration of almost all the macronutrients (except Ca), showed the highest concentrations of the surveyed micronutrients. This ratio of the accumulated elements resulted in the lowest accumulation of dry matter and a low synthesis rate of the photosynthetic pigments.

The obtained results confirmed the genotype specificity of mineral nutrition in sunflower cultivated in *in vitro* conditions. The next step should be the application of the acquired knowledge to develop genotype-specific media to enhance the regeneration potential of genotypes. A study is in due progress aimed at establishing possible differences between regenerating and nonregenerating calluses in the content of nutrients and biochemical activity within a single genotype. This study will give a comprehensive picture of the requirements for nutrients, of prospective genotypes grown in in vitro conditions, broadening the applicability of tissue culture methods in breeding programs.

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CARACTERÍSTICAS BIOQUÍMICAS Y EL CONTENIDO DE NUTRIENTES EN LOS CALLOS DE LAS LÍNEAS CONSANGUÍNEAS *(INBRED)* DE GIRASOL

RESUMEN

La nutrición mineral es un factor que influye en la regeneración *in vitro*. Diferentes requerimientos para ciertos elementos minerales que demuestran diferentes especies vegetales y diferentes genotipos de una misma especie, han llevado hasta la diferenciación y definición de la importancia de la base genética para la determinación de las características bioquímicas del callo y determinación de si diferentes requerimientos para los nutrientes dejan la posibilidad de logro de una mejor y más eficaz regeneración mediante unas modificaciones específicas en el contenido del substrato. El aspecto práctico de los resultados obtenidos, está en el hecho de que resulta baja la aptitud de regeneración, característica para las especies de girasol. Se ha mostrado que el dinamismo de aprobación y acumulación de los elementos minerales, es una característica que depende del genotipo, y que es más o menos visible dependiente de los elementos. El total contenido de nitrógeno era entre 4 y 5%, mientras que el genotipo del girasol PH-BC_2-101A mostró la mayor acumulación de nitrógeno, fósforo y hasta potasio, hasta cierta medida. Por lo tanto, se ha notado alta acumulación de la masa seca en totalidad, sin síntesis de pigmento. La alta acumulación de nitrógeno en dicho genotipo, llevó hasta la mayor actividad de reductasa de nitrato, lo que resultó en alta acumulación de proteínas solubles (enzimas).

CARACTÉRISTIQUES BIOCHIMIQUES ET CONTENU DE NUTRIMENT DANS LES CALLUS DES LIGNÉES DE TOURNESOL

RÉSUMÉ

La nutrition minérale est un facteur qui a un effet sur la régénération *in vitro*. Les différents besoins de certains éléments minéraux manifestés par différentes espèces végétales et différents génotypes de la même espèce ont conduit à la différenciation et à la définition de l'importance des bases génétiques dans la détermination des caractéristiques biochimiques du callus et dans la question de savoir si des besoins de nutriments différents laissent la possibilité d'atteindre une régénération meilleure et plus efficace à l'aide de modifications spécifiques dans le contenu du substrat. L'aspect pratique des résultats obtenus réside dans le fait que la faible aptitude de régénération est caractéristique du tournesol. Il a été démontré que la dynamique d'appropriation et d'accumulation d'éléments minéraux est une caractéristique dépendante du génotype et qui est plus ou moins apparente selon les éléments. Le contenu total d'azote était entre 4 et 5% alors que le génotype de tournesol PH-BC_2-101A a montré la plus grande accumulation d'azote, de phosphore et aussi de calcium jusqu'à une certaine mesure. Par conséquent une accumulation élevée de la masse sèche totale sans synthèse de pigment a été constatée. La grande accumulation d'azote dans le génotype mentionné a conduit à la plus grande activité de réductase de nitrate ce qui a résulté en une grande accumulation de protéines solubles (enzymes).