DIFFERENTIATION AND PROLIFERATION OF BASIL CALLUS TISSUE (*OCIMUM BASILICUM* L VAR. GREEA). OBSERVATIONS ON THE CONSISTENCY AND WEIGHT OF PLANT CALLUS MASS

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Abstract

*Seeds' germination potential was recorded on growth mediums MS1/2 (V_1); MS1/2+3g/lCV (V_2) and MS1/2 + 0,5mg/l AIA and BA (V_3), as well as proliferation capacity of the plant callus on growth mediums having 2.4D (**D_o**=MS+5mg/l2,4D, **D_1**= D_o+5mg/lBA, **D_2** =D_o+5mg/l2iP). Germination of basil seeds after 30 days: is very good, over 90% on V_1; 58% on V_2, with regeneration of a root plant/explants of circa 10 cm; 11% on V_3, with formation of 3-4 seedlings 1 cm tall, complete with roots (apex donor seedlings). Plant callus tissue's capacity to proliferate: **- after 33 days** the callus was differentiated, its diameter grew up to 0.9 cm Ø on the 2,4D only culture medium and twice as much on mediums also containing cytokines (1.8-2.0 cm Ø); **- after 62 days** the callus reached circa 3 cm in diameter, but consistency and friability levels suffer due to mild necrosis. Biomass obtained from callus after 33 days amounts to 0.1 gr/sample (wet mass) on medium D_o and circa 2 gr/sample (wet mass) on mediums enriched with cytokines (D_1 and D_2); after 62 days weight significantly increased to 3 gr/sample in D_1 and 2.5 gr/sample for D_2. The weight of basil callus tissue (after drying) after 33 days reached between 0.1 gr on D_o, 0.52 gr on D_1 and 0.39 gr on D_2; after 62 days the mass doubled: to 1.4 gr/sample on V_1, and 1.1 gr/sample on V_2.*

Keywords: *Ocimum basilicum* L, callus regeneration, apex, callus size (Øcm), rhizogenic callus

INTRODUCTION

Ocimum basilicum L is a species less studied in vitro. It is used in herbal medicine (in gastrointestinal and digestive disorders, as an anti-inflammatory and antiseptic etc.), in the fragrance and food industries (Păun I. et al. 1988). The volatile oil in basilicum can be found in proportion of 0.1-0.2%, and the chemical composition varies across species (Crăciun et al., 1977). It is an annual plant, a thermophile sensitive to frost, with high demands on light, humidity and soil (Păun I et al. 1986). It prefers the West and South of Romania: local improved varieties are grown here, as well as crop adapted foreign ones (Roman et al. 2012). This is a medicinal and aromatic herb used for its aerial parts that contain up to 1.5% volatile oils (depending on variety): citrate, linalool, camphor etc. (Bojor and Alexan, 1983). *Herba Basilici* is formed of stems and young branches covered in mostly green leaves, but also red-burgundy ones, as in the case of the variety we studied. *Ecology of the species*: light and heat loving plants, the richest in volatile oils of the Lamiaceae, it has moderate humidity demands and high soil demands, preferring rich permeable soils (Axinte et al 2006).

In our country the species is zoned in Baraganului, Burnazului, Olteniei and Timis plains (Berbecel and Valuță, 1960), but is also present in controlled crops. The implications of plant biotechnologies in the multiplication of some species are well known (Ungureanu, 1989; Cachiță et al 1997), as is the role of the callus tissue in species holding biological interest. The callus is a cellular mass with rapid division capacity and characteristic properties, that differentiates but only in the presence of a specific hormone balance given by the nature and dosage of hormones, that is critical in the differentiation of the tissue and that can lead to rhizogenesis, caulogenesis, and somatic embryogenesis (Cachiță et al., 2004). The proliferation of the callus in order to obtain increased mass depends on *the type of explants* used, which can be apex, leaf fragment, bud, hypocotyl segment, roots etc. (Petrescu and Cachiță, 1993; Li. J. et. al., 2004), *the species and its physiological state* (Sandu and Cachiță, 2000) and *the presence of auxin 2,4D in the culture medium* in a certain dosage and sometimes in combination with cytokines (Cristea and Deliu, 1993). The *in vitro* caulogenesis process can also be successfully completed with detached tissues from *in vitro* germinated seeds (Laslo, 2013) and from callus through somatic embryogenesis (Castillo et al., 2000). A large number of studies and research is concerned with the regeneration and *in vitro* breeding of some medicinal plants (cultivated or spontaneous) and with the isolation and analysis of substances extracted from the *in vitro* tissue obtained (Naika and Krishna, 2007). In addition, numerous studies have targeted the regeneration of species of medicinal, rare or endangered species from callus (Maruthi et al. 2004; Naika and Krishna, 2008). In this area, we find remarkable results concerning the selection and induction of regenerative callus in sugar cane (Sandu and Cachiță, 1994), as well as the characterizing of *in vitro* generated callus from different beetroot explants, on various aseptic mediums (Saunder, 1989; Sandu, 1996). In this context we also mention the reaction of explants from some medicinal plants (Menta piperita) to *in vitro* micropropagation (Laslo et al., 2011) and the proliferation capacity of *Arnica montana* callus, known for its phytopharmaceutical properties (Zăpârțan and Deliu, 2001; Zăpârțan 2001).

MATERIALS AND METHODS

Classic breeding technology for basil is through seedlings obtained from seed germination, which usually have a low germination value, under 40% after circa one month after sowing (Muntean, et. al. 2007). Basilicum seeds are rich in mucilage, which makes *in vitro* culture technology a little difficult (Cachiță D, 1987). In this experiment, we tested the *in vitro* germination capacity of these seeds on different medium formulas: V_1 = MS1/2; V_2 = MS1/2 + 3g/l CV (vegetal coal); and V_3 = MS1/2 +

0,5mg/lAIA + 0,5mg/lBA (Table 1). *Vegetal material:* after obtaining new seedlings from germinated seeds, the apex was removed and bred on three mediums (specified in Table 1), to stimulate the proliferation of callus tissue. These mediums contain 2,4D, either alone (**D_o** = MS + 5mg/l2,4D), or in combination with various cytokines in equal concentration (**D_1** = D_o + 5mg/ l BA; **D_2** = D_o + 5mg/l 2iP); they are presented in Table 2. After inoculation on mediums (10 repetitions/variant), the explants were kept in the conditions of the culture room, with a photoperiod of 16 hours of light and 8 hours of darkness, light intensity of 50µmol.m^{-2}.s^{-1}, for 62 days, constant temperature of 26^0C and relative humidity of 80%.

RESULTS AND DISCUSSION

Germination capacity of basilicum seeds (on mediums in Table 1) and *callus proliferation capacity on mediums with 2,4D* (in Table 2) were studied. Initially, observations were made regarding *the percentage of germinated seeds after 30 days* on the three medium variants and the evolution of *in vitro* seedlings after the same time period, as well as regarding the behavior of apex donor, *in vitro* formed new seedlings towards stimulating the process of callogenesis. Seeds were disinfected through immersion in 80^0 ethanol for 60 s, immersion in calcium hypochlorite for 15 min and 4 rinses with sterile water. The germination percentage obtained is very high: over 90% on V_1, resulting in 1 plant 1 cm tall with weak roots; on V_2 the percentage drops to half (circa 58%) but results in a rooted plant 10 cm tall; on V_3 germination is weak (circa 11%) but results in an average of 4 plants 1 cm high with roots and a callus sleeve at the base (Table 1). The germination capacity of seeds is shown in Figure 1 (percentage of an average of 100 germinated seeds/variant). The highest germination percentage of 57% is obtained on MS medium without hormones, with the amount of macro and micro elements halved, followed by the vegetal coal medium with 36%, and the phytohormones medium with 7%.

Table 1

Germination capacity of Ocimum basilicum L seeds, after 30 days
(MS1/2 = Murashige Skoog with macro + micro halved; CV = vegetal coal;
AIA = α indoleacetic acid; BA = benzylaminopurine)

Var.	Medium composition	Germin. %	Seedlings evolution	Observations
V_1	MS 1/2	92	1 sd. of 2,5 - 3,0cm With few roots	Very high germination % but seedlings are shorter and with frail root system
V_2	MS1/2 + 3g/lCV	58	1 sd of cca. 10cm with well developed root system	Lower germination % (only ½ of seeds): tall sd. with 6 pairs of actual leaves
V_3	MS1/2+0.5mg/lAIA + 0.5mg/l BA	11	2 - 4 sd. of 0,5 - 1cm, branched and well rooted	Seedlings have callus tissue at their base

The behavior of seeds placed in *in vitro* germination that we found is in agreement with that discovered by other researchers in the field (Cachiță, 1987; Cachiță et. all., 1997).

The proliferation capacity of the callus on mediums containing 2,4D was monitored from the point of view of consistency and color of basil callus, as well as the regenerative capacity of the callus obtained on medium cultures with 2,4D, from apical tissue, 33 and 62 days after inoculation on culture mediums, respectively (Table 2). Auxin 2,4D was administered either alone in a dosage of 5 mg/l (D_o), or in the presence of a cytokine with the same concentration: BA-5mg/l (V_1) and 2iP – 5 mg/l, the high dosages used with the intent of obtaining a highly friable consistent biomass from which the bioactive substances contained in basilicum callus could be extracted. The diameter of the callus mass *after 33 days* is between 0.9 and 2.0 cmØ, double this amount on variants with 2,4D and cytokines : the color of the callus is bright red and the consistency mildly friable in the presence of cytokines, and hard on mediums with 2,4D only. This type of evolution of callus consistency in medicinal plants on mediums with 2,4D, in the presence and absence of cytokines, has been studied before with great interest (Deliu, 2004). Part of the callus phials were analyzed for fresh and dry tissue weight (Table 3).

Table 2

Diferentiation and proliferation of callus tissue from Ocimum apex, depending on medium composition and incubation time

(MS = Murashige-Skoog; 2,4D = DDT; BA = benzylaminopurine: 2iP = 2-isopentenyladenin)

Var	Medium composition for callus differentiation	Incub. time	diam. (cm)	color	consistency	Regen. capac. (%)	Observations
Do	MS + 5mg/l2,4D	33 days	0.9	Greenish red	hard	0	No regenerative aspect
D1	Do + 5mg/ l BA		2.0	Bright red	mildly friable	58	Embryos on the callus surface
D2	Do + 5mg/l 2iP		1.8	Bright red	mildly friable	35	With embryos (white cones)
Do	MS + 5mg/l 2,4D	62 days	1.0	Bright red mildly necrotic	Hard, hints of necrosis	-	Necrotic 45%
D1	Do + 5mg/l BA		3.2	Bright red mildly necrotic	Friable	-	Callus for suspensions
D2	Do + 5mg/l2iP		2.9	Bright red mildly necrotic	Friable	-	Callus for suspensions

Of particular importance is the regenerative aspect of the callus after this period of time, which will be followed with care and will determine future conduct in order to obtain new seedlings: usually, embryogenic callus will quickly be transferred on fresh mediums without 2,4D and with an increased amount of phytohormones for plant differentiation (Cachiță and Ardeleanu 2009). After another month, *62 days from tissue incubation*, the diameter of the callus mass grows considerably, reaching over 3 cm Ø on mediums with cytokines, but necrosis is signaled on the contact area with the culture medium, as well as friability and color changes (the seedling gets a brownish hue). Embryos retreat, and so do the growth cones (meristems), the first phase in seedling regeneration (this tissue appeared after 33 days

and was recorded in Table 2). After 33 and 63 days, respectively, from callus tissue differentiation, fresh and dry callus mass is recorded. Average values appear in Table 3, alongside variantvalues on this parameter (through bonification). Following the table data we can observe that on D_1 with 5mg/lBA and on D_2 with 5mg/l 2iP callus tissue reaches maximum growth.*Fresh and dry weight of basilicum callus*: was determined in order to obtain a biomass with the species' characteristic active properties and then analyze said biomass for phytopharmaceutical purposes.

Table 3

In vitro evolution of basil callus (Ocimun basilicum L. var. Greea) on mediums with 2,4D (after 33, and 62 days respectively)

Incubation period	Var.	Ø of callus mass	Weight fresh (gr.)	Weight dry (gr.)	Bonification
33 days	D0	0.9 ± 0.08	0.1250	0.0100	xxx
	D1	2.0 ± 0.16	1.9053	0.5115	xxxxxx
	D2	1.8 ± 0.14	1.8209	0.3981	xxxxxx
days	D0	1.0 ± 0.09	0.1950	0.0508	xxx
	D1	3.2 ± 0.24	3.0399	1.3902	xxxxxx advanced ev.
	D2	2.9 ± 0.18	2.5409	1.0990	xxxxxx

We note that the maximum fresh weight is reached after 62 days and amounts to 3.0 gr/sample, and 2.5 gr/sample respectively, on variants with 2,4D and added cytokines (D_1 and D_2). Dry weight reaches maximum values in these same variants, at 1.4gr/sample on D_1 and circa 1gr/sample on D_2. On the medium containing benzyladenine (D_1) callus values are higher. After two months of callus growth on mediums with 2,4D we observed degradation in the callus through necrosis and changes in tissue consistency, as the tissue has to be moved to fresh solid mediums for further callus proliferation , or to liquids for cell suspensions (Deliu, 2004). Differentiated embryogenic callus after 33 days (Table 2) also starts to degrade after a while through the disappearance of growth cones, therefore it has to be moved after this time on fresh mediums, without 2,4D and with a balanced amount of phytohormones (cytokines and auxins) for new seedlings to differentiate.

CONCLUSIONS

1. Basilicum seeds *germinate* very well on a simple medium (MS1/2) and on vegetal coal medium V_2 and weakly on a hormone enriched medium (V_3): on V_2 results a new seedlingof circa 10 cm, very well developed, and on V_3 circa 4 new seedlings, well built and apex donating (explants for initiating callus tissue).
2. One month (33 days) after incubation of the apex on mediums with 2,4D, *a callus mass differentiates* of 0.9cmØ on D_o and double that amount, 2.0cmØ and 1.9cmØ respectively, on variants with cytokines ($D_1$and D_2);

3. After 62 days (another month) the callus mass on variants with 2,4D and cytokines reaches 3.0cmØ, but consistency and color suffer mild degradation;
4. We recommend medium MS1/2 for seed germination, while for callus differentiation medium MS+2,4D+cytokines;

Photos 1 and 2 Differentiated plants from germinated seeds

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