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CROP PRODUCTION

Micropropagation of basil 'Grecco a Palla' mediated by 6-benzylaminopurine and 3-indole butyric acid

Felipe Gorski[1] Geysiane Moreira Gerotti[1] Hélida Mara Magalhães[1*]

[1]Laboratório de Cultura de Tecidos Vegetais, Universidade Paranaense (UNIPAR), 87502-210, Umuarama, PR, Brasil. E-mail: hmaramagal@gmail.com. *Corresponding author.

ABSTRACT: The present study evaluated the efficiency of a protocol for micropropagation of stem apexes and nodal segments of basil 'Grecco a Palla' in various concentrations of 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA). A completely randomized design was used with six treatments distributed in five replications. A medium without growth regulators favored the survival of *Ocimum basilicum* stem apexes inoculated *in vitro*, and thereby promoted the sprouting of explants, whereas, for nodal segments, it was necessary to use regulators, and the concentration of 0.5 $mg.L^{-1}$ BAP 0.0 $mg.L^{-1}$ of IBA was more beneficial for the species.
Key words: auxin, cytokinin, *in vitro* culture, Lamiaceae, *Ocimum basilicum* L.

Micropropagação de manjericão 'Grecco a palla' mediado por 6-benzylaminopurine e 3-indole butyric acid

RESUMO: O objetivo deste trabalho foi avaliar a eficiência de um protocolo de micropropagação de ápices caulinares e segmentos nodais da cultivar de manjericão (*Ocimum basilicum* L.) 'Grecco a palla' em diferentes concentrações de BAP (6-benzilaminopurina) e de AIB (ácido 3-indol butírico). Foi utilizado delineamento experimental inteiramente casualizado, com seis tratamentos distribuídos em cinco repetições. Para ápices caulinares, meio sem a adição de reguladores de crescimento favoreceu a sobrevivência de ápices caulinares de *O. basilicum* inoculados *in vitro*, promovendo a brotação dos explantes. Enquanto que para segmentos nodais houve necessidade do uso de reguladores, sendo que a concentração de 0,5 $mg.L^{-1}$ de BAP e 0,0 $mg.L^{-1}$ de AIB foi mais benéfica para a espécie.
Palavras-chave: auxinas, citocininas, cultivo *in vitro,* Lamiaceae, *Ocimum basilicum* L.

Basil is a native plant reported in Africa, Asia, and the Pacific Islands and is widely grown in the tropical regions. The plant is herbaceous, annual, and is widely used in folk medicine mainly because it contains several phenolic compounds and flavonoids that act as potential antioxidants (BATSATSASHVILI et al., 2020).

In vitro culture is majorly used to produce plants with the desired trait. Cultivation using stem apexes and nodal segments is better for micropropagation resulting in a higher rate of true plants with multiple shoots (JOHNS, 2019). The objectives of *in vitro* cultivation include: the development of low-cost protocols, associated with lesser use of chemicals, and associated with a high rate of multiplication, rooting, and plant survival (SINGH, 2015).

The present study investigated the effect of different concentrations on plant growth regulators (PGRs) 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA) in combination on the micropropagation of stem apexes and nodal segments of *Ocimum basilicum* 'Grecco a Palla'. Moreover, to evaluate this PGRs intended to evaluate and the explant type to be used in future experiments.

Seeds of *O. basilicum* from cultivar 'Grecco a Palla' were commercially purchased from Isla Sementes® (Porto Alegre, Brazil) (lot number 110876 with 100% purity and 86.0% germination). Thereafter, they were disinfected in laminar flow and kept for 2 min in 70% ethyl alcohol. They were then added in a 2% sodium hypochlorite solution for 15 min, with stirring. Subsequently, four successive washes were performed with sterile deionized and autoclaved 60 days of *in vitro* culture. These served as donors of stem apexes and nodal segments. The apexes had an average of 1 cm length and had two cotyledons. The length of nodal segments also had was 1 cm and had two leaf t were removed from the third median seedling third. The explants were inoculated in 350

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ml flasks containing 50 ml of Murashige and Skoog (MS) culture medium (MURASHIGE & SKOOG, 1962), in total salt concentration, supplemented with 30 $g.L^{-1}$ of sucrose and 6.5 $g.L^{-1}$ of agar with pH adjusted to 5.8. All chemicals were purchased from *Sigma-Aldrich*.

Two experiments were carried out independently. In the first experiment, the stem apexes were inoculated in a medium supplemented with six BAP concentrations along with five IBA concentrations, i.e., A1: 0.0, 0.0; A2: 0.1, 0.0; A3: 0.5, 0.0 A4: 0.1, 0.05; A5: 0.05, 0.05; A6: 0.5, 0.1 $mg.L^{-1}$ of BAP and IBA, respectively. In the second experiment, the nodal segments were inoculated in a medium supplemented with six concentrations of regulators, i.e., being NS1: 0.0, 0.0; NS2: 0.1, 0.0; NS3: 0.5, 0.0; NS4: 0.1, 0.05; NS5: 0.05, 0.05; NS6: 0.5, 0.1 $mg.L^{-1}$ of BAP and IBA, respectively. These concentrations were based on previous studies (TRETTEL et al., 2018). Both experiments were conducted in a completely randomized design (CRD) with six treatments and five replications. Each replication consisted of six flasks containing one explant.

The culture medium was autoclaved for 20 min at 120 °C, under 1 atm. After inoculation, the explants in the flasks, are closed with plastic caps and sealed with parafilm. The flasks were kept in a growth room with a 24 – hour photoperiod using Blumenau® light emitter diodes (LEDs), LED T8 10W 6,00 K, 100-–40 V-–0/60 Hz, power factor: ≥0.92 (High PF), at a temperature of 25 ºC± 2 ºCand light intensity of 72.02 $\mu mol\ m^{-2} \cdot s^{-1}$.

At 60 days, the number of shoots (NS), number of leaves (NL), root length (RL) (in mm), shoot length (SL) (in mm), root fresh matter (RFM) (in g), shoot fresh matter (SFM) (in g), relative chlorophyll index (RCI), relative phenol index (RPI), and anthocyanin relative index (RAI) were evaluated. The parameters SL and RL were determined with the aid of a digital caliper. The shoots were measured from the base of the sprout to the apical bud. In contrast, RCI, RPI, and RAI were determined from fresh leaves using the equipment DUALEX SCIENTIFIC+ ™ Quick Start model (FORCE A®, France, Paris) from manufacturer's instructions with six replications per treatment. Three fully expanded leaves were evaluated in each replication.

The data obtained from the tissues under the different treatments were submitted to the normality test by Shapiro Wilk ($P < 0.05$). After which, the variance was carried out, using the F test ($P < 0.05$). The average values were compared using the Tukey's test ($P < 0.05$). The Sisvar® software version 5.6 (FERREIRA, 2011) was used for all analyzes. The PGR concentrations used influenced the traits evaluated in various ways at the end of 60-day cultivation of stem apexes and nodal segments of basil. Callus formation was not observed in any treatment.

Stem apexes inoculated without PGRs, A1, produced more vigorous seedlings (Table 1). These seedlings presented RL and RFM values that were 15% and 17% higher than those of the A2, respectively. One of the desired traits of *in vitro* micropropagation, the emission of new roots was favored in A1. This presumably contributed to the other traits; with more established roots, it was possible to observe higher values of NL and SL. When compared to treatment showing the second-best results, A1 presented values of NL 12% higher than A6, SL 30% higher than A4, and SFM 23% higher than A2. Using the total concentration of salts in the MS medium can contribute to this rooting and subsequent increase in shoots, providing the necessary nutritional supply for seedling growth (ASSAF et al., 2022). This has already been reported for other basil cultivars, such as 'Red Rubi' (DA SILVA et al., 2017) and 'Genoves' (TRETTEL et al., 2018).

The results found for stem apexes in the medium without PGRs this may indicate that endogenous auxin present during the explant extraction was sufficient to promote growth. It has been previously reported that the main production sites of auxin are apexes of juvenile shoots (BRUMOS et al., 2018). This phytohormone stimulates the development of root founder cells (MÖLLER et al., 2017). The synthesis of cytokinin is presumably initiated from the root formation and establishment since this organ is its main synthesis site (SINGH, 2019).

The addition of regulators in A6 treatment had a deleterious effect on the RL and RFM traits compared to A1, was and 80% reduction in RL and 8FM, respectively. The culture media were inoculated with BAP, except for A1, with A6 having the highest concentration, of 0.0 $mg.L^{-1}$. Cytokinin signaling regulates the initiation of lateral roots (JING & STRADER, 2019); this my indicatethat, by hindering the polar transport of auxin, it inhibits the signaling of the root meristem and the initiation of the root (DU & SCHERES, 2018).

Contrary to what was observed in stem apexes, at 60 days, it was possible to observe changes in the growth of nodal segments (Table 2). In a culture medium containing BAP stimulated seedling growth,

Table 1 - Average number of shoots (NS), average number of leaves (NL), root length (RL), shoot length (SL), root fresh matter (RFM), shoot fresh matter (SFM), relative chlorophyll index (RCI), relative phenol index (RPI), and relative anthocyanin index (RAI), obtained from stem apexes of *Ocimum basilicum* 'Grecco a Palla' grown under different concentrations of 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA).

Treatment	NS	NL	RL (mm)	SL (mm)	RFM (g)
A1	8.40±2.07^{b*}	135.00±10.95^{a}	88.38±12.33^{ab}	74.59±11.60^{a}	12.0173±1.60^{a}
A2	5.00±1.00^{c}	92.00±18.54^{b}	75.24±13.76^{bc}	63.70±7.92^{a}	10.1141±2.78^{ab}
A3	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
A4	7.00±0.00^{b}	78.00±11.31^{b}	97.02±6.76^{a}	53.15±19.73^{ab}	6.1624±3.91^{bc}
A5	3.66±0.57^{c}	151.67±11.93^{a}	47.05±3.95^{d}	51.33±8.56^{b}	4.6637±0.30^{c}
A6	18.33±1.52^{a}	119.67±3.78^{a}	62.86±12.67^{cd}	49.65±4.57^{b}	2.0605±0.03^{d}
Treatment	SFM (g)	RCI	RPI	RAI	
A1	11.3971±0.42^{a}	31.46±1.90^{a}	0.45±0.04^{a}	0.27±0.03^{a}	- - -
A2	8.8207±1.14^{b}	0.94±2.96^{d}	0.30±0.07^{b}	0.26±0.01^{a}	- - -
A3	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}	- - -
A4	5.5346±4.37^{cd}	9.78±2.57^{c}	0.20±0.02^{c}	0.23±0.02^{ab}	- - -
A5	6.5975±1.00^{c}	13.55±2.47^{c}	0.27±0.03^{b}	0.21±0.02^{b}	- - -
A6	4.1073±0.10^{d}	20.95±2.66^{b}	0.24±0.02^{b}	0.20±0.03^{b}	- - -

*Means ± standard deviation followed by the same letters in the column do not differ statistically by Tukey's test ($P \leq 0.05$). A1: 0.0, 0.0; A2: 0.1, 0.0; A3: 0.5, 0.0; A4: 0.1, 0.05; A5: 0.05, 0.05; A6: 0.5, 0.1 mg.L^{-1} of 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA), respectively.A3 oxidation of all explants.

as verified in NS3 at a concentration of 0.5 and 0.0 mg·L^{-1} of BAP and IBA, respectively. This treatment presented NS and SL values, 62% and 25% higher than those found for NS1, respectively. Considering the roots, NS3 presented values of RL and RFM 29% and 33% higher than NS1, respectively. Therefore, notably, the shoot development involved in this study was enhanced by cytokinin since it is one of the most important PGRs influencing cell division and shoot induction. In *in vitro* cultivation PGRs played a crucial role in the regeneration of explants, as they have an inducing effect on the formation of shoots (HAILEKIDAN et al., 2013). These may have led NS3 to present the highest values for the indexes previously mentioned.

Furthermore, the promoter effect of cytokinin on shoots regeneration has been reported in species of the *Ocimum* genus. Using nodal segments from two *O. tenuiflorum* cultivars in MS medium, SAHA et al. (2016) reported the greatest regeneration of explants, and the largest root formation in the medium containing only BAP. For *O. tenuiflorum*, purple leaf cultivar, the highest rates were same as in this study, i.e., 0.5 mg.L^{-1}. Nonetheless the 'Basilicão' cultivar, the highest rates were found with the BAP concentration of 1.0 mg.L^{-1}.

Stem apexes without the use of RCV have the highest IRC value. The A1 showed a value 67% higher than A6, which obtained the second average and 98% higher than A6, which was the lowest average. This is probably because exogenous PGRs normally delay the entry of water into plant cells. Moreover, the treatments where a decrease in RCI revealed a tendency to reduce SL and SFM. Plants with higher chlorophyll indexes are less susceptible to oxidative stress due owing to defects in the photohsynthetic apparatus (TOLAY, 2021). The values of RPI and RAI in both explants presented higher values in the culture media without PGRs. In stem apexes, RPI in A1 was 45% higher than A4. In nodal segments, NS1 was 48% higher than NS4. About RAI in stem apexes, A1 was 42% higher than A6, and simultaneously, NS1 was 64% higher than NS6.

These results demonstrated the basil's 'Grecco a Palla' potential in phenol production. One of the factors that can be attributed to this is the root and shoot generation in these treatments since the formation of specialized tissues is a prerequisite for producing these compounds in vegetables (GÓRSKI et al., 2021). The number of phenols is also related to organogenesis regulation in the area, i.e., appropriate development leads to higher phenol rates (JAKOVLJEVIĆ et al., 2022).

Table 2 - Average number of shoots (NS), average number of leaves (NL), root length (RL), shoot length (SL), root fresh matter (RFM), shoot fresh matter (SFM), relative chlorophyll index (RCI), relative phenol index (RPI) and relative anthocyanin index (RAI), obtained from nodal segments of *Ocimum basilicum* 'Grecco a Palla' grown under different concentrations of 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA).

Treatment	NS	NL	RL (mm)	SL (mm)	RFM (g)
NS1	6.50±0.70^{c*}	167.5±98.00^{ab}	84.41±44.44^{b}	85.73±8.70^{b}	7.9200±2.13^{b}
NS2	13.33±4.50^{b}	92.00±33.06^{b}	108.74±9.46^{a}	72.09±8.38^{c}	7.9054±2.49^{b}
NS3	17.00±1.41^{a}	127.5±68.68^{ab}	103.60±2.60^{a}	115.04±18.78^{a}	11.7913±0.34^{a}
NS4	10.50±2.12^{b}	94.00±7.07^{b}	90.04±35.41^{ab}	77.06±8.86^{b}	4.6167±0.37^{c}
NS5	12.66±1.15^{b}	132.67±22.23^{a}	71.05±7.92^{b}	62.82±6.28^{c}	6.9792±2.72^{bc}
NS6	18.66±5.50^{ab}	59.00±14.52^{c}	107.26±15.10^{a}	83.10±3.70^{b}	2.1651±0.16^{d}
Treatment	SFM (g)	RCI	RPI	RAI	
NS1	10.9957±2.87^{b}	11.53±2.23^{b}	0.48±0.04^{a}	0.28±0.03^{a}	- - -
NS2	7.3360±2.49^{d}	13.02±2.49^{ab}	0.46±0.11^{ab}	0.26±0.03^{a}	- - -
NS3	11.0190±2.22^{b}	11.33±1.26^{b}	0.25±0.02^{c}	0.24±0.01^{ab}	- - -
NS4	14.5329±0.60^{a}	11.46±1.34^{b}	0.20±0.02^{c}	0.22±0.01^{b}	- - -
NS5	9.8110±1.40^{bc}	16.22±2.91^{a}	0.41±0.07^{b}	0.26±0.02^{a}	- - -
NS6	13.4195±5.85^{ab}	11.35±1.90^{b}	0.28±0.02^{c}	0.18±0.03^{c}	- - -

*Means ± standard deviation followed by the same letters in the column do not differ statistically by Tukey's test ($P \leq 0.05$). NS1: 0.0, 0.0; NS2: 0.1, 0.0; NS3: 0.5, 0.0; NS4: 0.1, 0.05; NS5: 0.05, 0.05; NS6: 0.5, 0.1 mg.L^{-1} of 6-benzylaminopurine (BAP) and 3-indole butyric acid (IBA), respectively.

This study presented a method for *in vitro* regeneration of basil 'Grecco a Palla' from stem apexes and nodal segments. The standardization of this protocol may present a promising strategy for the regeneration of these explants to guarantee the formation of uniform plants enabling its use in producing secondary metabolites for research in medicine or subsequent genetic studies. Basil 'Grecco a Palla' stem apex micropropagation was favored in A1, without the addition of PGRs. Meanwhile, the micropropagation of nodal segments required the use of regulators, where NS3 favored growth with a concentration of 0.5 mg.L^{-1} of BAP and 0.0 mg.L^{-1} of IBA.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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