Escuela Agrícola Panamericana, Zamorano

Department of Science and Agricultural Production

Agronomic Engineering

Special Graduation Project

Evaluation of plant growth regulators in the *in vitro* multiplication stage of basil *Ocimum basilicum L.*

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Honduras, July 11, 2022

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Resumen

A lo largo de los años se ha encontrado que la albahaca *(Ocimum basilicum L.)* tiene un gran potencial económico gracias a sus características únicas en la industria medicinal y culinaria. Sin embargo, existen pocos estudios que establezcan un protocolo óptimo para una micropropagación exitosa. Esta investigación tuvo como objetivo evaluar los efectos del ácido indol-3-acético (IAA) y 6-benzyadenina (BA) en diferentes concentraciones durante la multiplicación in vitro de las variedadeses de albahaca Genovese y Red rubin. Las yemas axilares y apicales de los explantes fueron extraídas y establecidas en medio de cultivo Murashiage y Skoog sin reguladores de crecimiento. A los 21 días los explantes establecidos se trasladaron a la etapa de multiplicación donde se evaluaron cinco tratamientos: medio sin regulador de crecimiento, 0.25 mg/L de BA, 0.5 mg/L de BA, 0.25 mg/L de BA + 0.01 mg de IAA y 0.5mg/L de BA + 0.01mg de IAA. Se analizó la formación de hojas, raíces, brotes y callos los días 21 y 42 en multiplicación. En esta etapa, los tratamientos con BA resultaron ser mas efectivos, permitiendo la mayor formación de hojas y brotes para ambas variedadeses. El crecimiento de la raíz fue más significativo en un medio libre de PGR y el callo fue dominante en los medios suplementados con PGR. En la variedad Red Rubin, la multiplicación sería mejor en un medio libre de reguladores de crecimiento. La albahaca genovesa tuvo efectos significativos con el tratamiento BA en la formación de brotes después de 42 días de multiplicación.

Palabras clave: Brotes, explantes, micropropagación, reguladores de crecimiento.

Abstract

Over the years it has been found that basil (*Ocimum basilicum* L.) has great economic potential thanks to its unique characteristics in the medicinal and culinary industry. However, there are few studies that establish an optimal protocol for a successful micropropagation. This research aimed to evaluate the effects of indole-3-acetic acid (IAA) and 6-benzyadenine (BA) at different concentrations during *in vitro* multiplication of the Genovese and Red Rubin basil varieties. The axillary and apical buds of the explants were extracted and established in a Murashiage and Skoog culture medium without growth regulators. The buds established at 21 days were transferred to the multiplication stage where five treatments were evaluated: plant growth regulator-free medium, 0.25mg/L of BA, 0.5mg/L of BA, 0.25mg/L of BA + 0.01mg of IAA and 0.5mg/L of BA + 0.01mg of IAA. The formation of leaves, roots, shoots, and calluses was analyzed on days 21 and 42 in multiplication. At this stage, BA was the most successful, allowing the formation of leaf and shoots for both varieties. Root growth was more significant in a PGR-free media and callus was dominant in PGR-supplemented media. The treatments supplemented with BA were found to be the most effective to induce the highest number of leaves in both varieties. In the Red Rubin variety, multiplication would be better off in a PGR-free medium. Genovese basil had significant effects with the BA treatment in shoot formation after 42 days in multiplication.

Keywords: explants, micropropagation, plant growth regulators, shoots.

Introduction

Basil (*Ocimum basilicum* L.) is a culinary, medicinal, and ornamental herb used around the world for its aromatic and stimulating properties. This herb is native to India and is highly cultivated in Asia, Africa, South America, and the Mediterranean. It is an annual herbaceous plant and belongs to the Lamiaceae family. For trade, there are three types of basil: the French, which is known to have the highest value, the American, distinguished by its high quality, and the European, seen as the most economical in terms of costs. With more than 50 species, basil is cultivated and famed for its fragrant leaf. Its species are characterized by their growth habits and aromatic, physical, and chemical compositions of each one, its quality is determined by its color and aroma (DAFF 2012). Most of the commercial basil varieties belong to the species *O. basilicum* and amongst them, there is the Genovese Basil, an Italian strain internationally known for its impact in the culinary industry and Red Rubin basil, grown for its ornamental potential thanks to its attractive purple foliage as well as its culinary use (Makri and Kintzios 2008).

The conventional method of propagating basil is via seed, however, the loss of seed viability after being stored for months results in low germination, restricting its multiplication.

Basil requires warm conditions for successful development. They are best grown in subtropical areas as they are susceptible to low temperatures. The optimum temperature for its growth is 20°C, increasing to 27°C (DAFF 2012). Likewise, there are diseases that easily affect the crop when the presence of humidity is remarkably high, this leads to a loss of yield and damage to the leaves. When handling seeds or vegetative parts that are contaminated, the pathogens present spread, resulting in fatal diseases for the plant (Tan 2011). Therefore, a sanitization of all the materials used in the propagation area is required.

As the years go by, there is an increasing interest in using techniques such as micropropagation or *in vitro* propagation to propagate at a faster and more efficient rate.

Micropropagation is an alternative that brings many benefits since we can produce a mass number of plants that are free of pathogens, all year long (Gaba 2005). The micropropagation technique is divided into five stages: 0) Mother plant selection I) Establishment, II) Multiplication of shoots or embryos, III) Rooting and IV) Acclimatization (Olmos et al. 2010). Of these five, stage II was evaluated. The multiplication of shoots and its efficiency is solely influenced by a plants genotype, explants and subculture, media composition, and *in vitro* environmental factors (Dobránszki and da Silva 2010). Generally, for the *in vitro* propagation of basil, axillary and apical meristems are used, these are located in the axil of each leaf, which are then separated to obtain good quality explants (Arévalo Ayala 2017).

On the other hand, despite its importance in the medical and culinary industry, there are very few studies on basil and its production, especially *in vitro* propagation (Trettel et al. 2018). Likewise, Ahmadi et al. (2013), shows that conventional propagation methods delay the reproduction of seedlings when exposed to biotic and abiotic factors, making it difficult to obtain high quality seedlings.

In tissue culture, *in vitro* propagation goes hand in hand with inclusion of plant growth regulators (PGRs) as they influence many physiological processes during plant development. Growth regulators help guide the direction, type, and amount a plant will grow. These molecular components are used commercially to promote vigorous growth and root formation (Morshed et al. 2009). When using the right concentrations of PGRs, especially of the combination of cytokinin and auxin, the dormancy of the axillary buds can be broken, resulting in the development of new shoots. Furthermore, the explants have the potential of multiplying at a fast rate when cultured in a media with optimum concentrations.

For the elongation of stems and inter nodes, rooting, apical dominance and tropism, auxins are generally supplemented to ensure success (Sharma et al. 2015). Indole acetic acid (IAA) is the most

common and abundant plant growth regulator of the auxin class, derived from plants and microorganisms. IAA impacts plant development and growth, triggering cell orientation, cell elongation, organ development and fertility (Labeeuw et al. 2016). Moreover, IAA must be used at low concentrations to avoid physiological damage due to its ability to stimulate the synthesis of ethylene, resulting in an inhibition of plant growth (Hong et al. 1996).

Cytokinins are another main group of plant growth stimulator typically used in cultured tissues that help determine the development of the plants. These growth regulators are best known for cell division, breakage of apical dominance of meristems, induction of axillary and adventitious shoot formation. A well-known type of cytokinin that is applied in tissue culture is 6-benzyladenine or BA. BA is commonly used at low concentrations to promote cell division and shoot differentiation in plant tissue culture (Mangena 2020). Accordingly, it was been known that BA has a significant effect on shoot formation and as its concentration in a medium increases, shoot formation does too (Asghari et al. 2012).

The evaluation of the effect of two plant growth regulators at different concentrations will help determine which is most successful to induce leaf, bud, root, and callus formation in the Genovese and Red Rubin varieties. The objective of this study will be to evaluate the effect of 6-Benzyladenine (BA) and Indole-3-acetic-acid (IAA) in the stage of *in vitro* multiplication of Genovese and Red Rubin basil.

Materials y Methods

The study was carried out in the Plant Tissue Culture Laboratory, located in the Department of Science and Agricultural Production of the Escuela Agricola Panamericana, Zamorano, Honduras.

Plant Material

The nodal segments containing apical and axillary buds were pruned from potted basil plants of the Red Rubin and Genovese variety (*Ocimum basilicum L.*). The Genovese basil contained dark green leaves and had a deeply cupped spoon shape and the Red Rubin variety had dentate oval leaves that stood out horizontally, with a deep purple color. The plants were 60 days old when pruned in the Plant Propagation Unit of Zamorano.

Disinfection of Plant Material

Large leaves were removed from the cuttings (Figure 1) and then washed thoroughly with tap water and soap. To disinfect its surface, the cuttings were soaked in for 5 minutes in a solution containing commercial chlorine (NaClO 4.72% active ingredient) at a concentration of 5% volume/volume plus two added drops of Tween® 80 for every 100 mL of the total solution. Afterwards, three washes were made with sterile distilled water inside the laminar flow chamber to prevent contamination.

Figure 1

Recollection and preparation of plant material

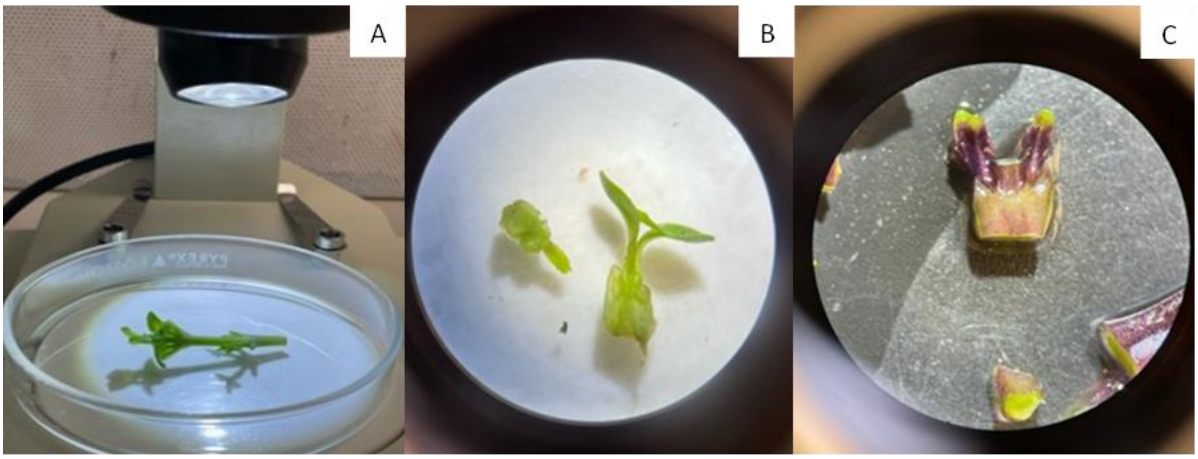
Note. (A) Genovese Basil (B) Red Rubin (C) Removal of large leaves (D) Separation into nodal segments.

Stage I (Establishment)

The apical and axillary buds of the nodal segments were separated (Figure 2) and established in a plant growth regulator-free Murashige y Skoog (MS) medium (Table 1) given that it has been evaluated that there are no significant differences in the addition of plant growth regulators in the establishment of basil (Arévalo Ayala 2017). The pH of the medium was adjusted with KOH and HCl to 5.8 before the Phytagel (gelling agent) was added. All media was then sterilized by autoclave at 121°C, 1 Kg/cm², for 20 minutes. In the laminar flow hood (purifier horizontal clean bench), the explants were introduced on the sterile medium for plant establishment.

Figure 2

Extraction of axillary and apical buds



Note. (A) Genovese Basil (B) Red Rubin (C) Removal of large leaves (D) Separation into nodal segments.

Table 1

Murashige and Skoog modified basal medium for the in vitro establishment of basil

Components	Formula	Common name	mg/L
Macroelements	NH_4NO_3	Ammonium nitrate	1650.000
	KNO_3	Potassium nitrate	1900.000
	$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate	370.000
	$CaCl_2.2H_2O$	Calcium chloride bihydrate	440.000
	KH_2PO_4	Monobasic potassium phosphate	170.000
Microelements	H_3BO_3	Boric acid	6.200
	$CoCl_2.6H_2O$	Cobalt chloride hexahydrate	0.025

Components	Formula	Common name	mg/L
Microelements	$CuSO_4.5H_2O$	Copper sulfate pentahydrate	0.025
	KI	Potassium iodide	0.830
	$MnSO_4.4H_2O$	Manganese sulfate tetrahydrate	22.300
	$Na_2MoO_4.2HO$	Sodium molybdate bihydrate	0.250
	$ZnSO_4.7H_20$	Zinc sulfate heptahydrate	8.600
	FeNa EDTA	Ethylenediaminetetraacetic acid ferric sodium salt	50.000
Vitamines		Inositol	100.000
		Thiamine	0.400
		Pyridoxine	0.500
		Nicotinic acid	0.500
Carbohidrates		Sucrose	30000.000

Note. from CIAT (1991)

Stage II (Multiplication)

After 21 days in establishment, the explants were transferred to the multiplication stage. The explants had visible growth overall where roots, callus, shoots and leaves were present. Before being transferred, large leaves, roots and callus was removed from the explants, regenerated shoots were separated and planted next to the original explant. The modified Murashige and Skoog medium was supplemented with growth regulators to evaluate its effect. The treatments evaluated were five (Table 2).

Table 2

Growth regulators supplemented in MS medium for basil in vitro multiplication

Treatment	BA mg/L	IAA mg/L
Control	0.00	0.00
BA25	0.25	0.00
BA5	0.50	0.00
BA25 + IAA	0.25	0.01
BA5 + IAA	0.50	0.01

Note. ¥BA: 6-Benzyadenine, 25=0.25mg/L; 5=0.5mg/L, IAA: Indole-3-Acetic-Acid 0.01mg/L

After 21 days in incubation, a refreshment of media was made, and the explants were subcultured in a new media of the same composition, this to rejuvenate the explants and promote shoot production (Dobránszki and da Silva 2010). Likewise, subculturing was executed to avoid

vitrification in the explants as they were transferred to a fresh medium. During this process, new formed buds were separated from the explant as well as large leaves, roots, and callus.

Incubation

During establishment and multiplication, cultures were incubated in a growth room under a controlled environment with a temperature of 25°C, a relative humidity of 30%, with a 16-hour photoperiod at 40 µmol/m²/second provided by cool white bulbs.

Variables Evaluated

In the multiplication stage, the number of shoots and leaves along with the percentage of roots and callus was evaluated after 21 days and then after 42 days as a final data collection.

Experimental Design and Statistical Analysis

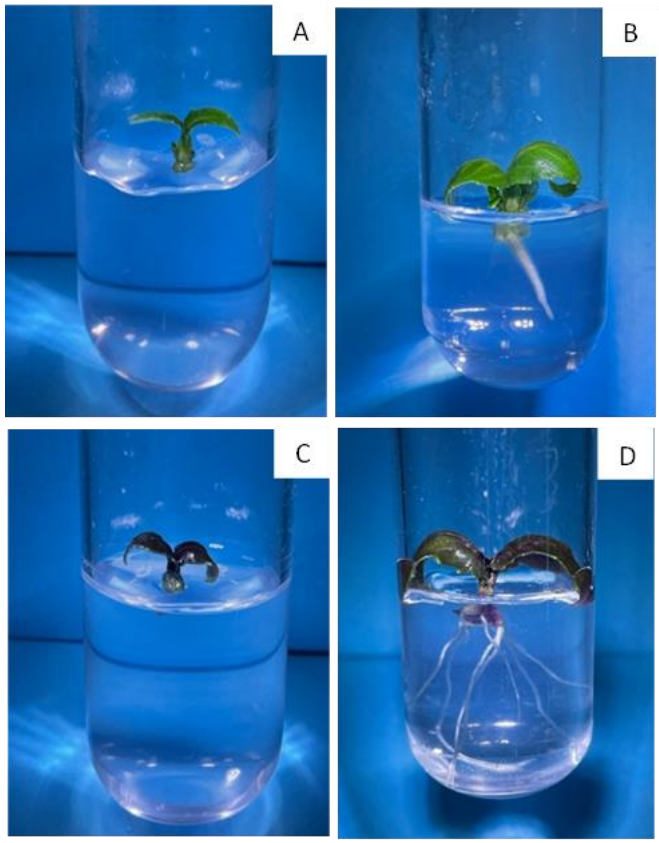
A completely randomized design (CRD) was made where five treatments were repeated three times with a total of 60 repetitions per treatment. The data was analyzed through an analysis of variance (ANOVA) and the difference between means were calculated using Duncan's Multiple Range Test ($p < 0.05$) through the statistical software program InfoStat.

Results and Discussion

Explants in the establishment stage had an exceptionally low rate of contamination with only 2% being discarded. A total of 98% of aseptic cultures transferred to stage II of *in vitro* propagation (Figure 3).

Figure 3

Stage I: Establishment of O. basilicum explants in a PGR-free medium



Note. Genovese basil (A) Day 7 (B) Day 21. Red Rubin (C) Day 7 (D) Day21.

Effects of Growth Regulators BA and IAA in Multiplication: Callus and Roots

In both varieties, the formation and expansion of callus was active in mediums with PGRs. In some, callus formation initiated earlier than others. Hence, the amount of callus was greater in treatments with BA and IAA combined, but overall, callus was present in all. Callogenesis originated at the base of the explants about 14 days after being cultured. Nazir et al. (2020) also report that

exogenous auxin and cytokinin are of great influence for callus induction. The presence and combination of the two PGR maximize cell division, stimulating callus production (Table 3). However, for in vitro multiplication, the treatments with no added plant growth regulators would be most beneficial given that we seek a greater number of shoots per explant, than callus per explant.

Table 3

Effect of growth regulators on the callus on days 21 and 42 at the multiplication stage of Genovese Basil and Red Rubin, percentage of explant with callus formation

Treatment[¥] (mg/L)	Genovese		Red Rubin	
	Callus day 21 (%)	Callus day 42 (%)	Callus day 21 (%)	Callus day 42 (%)
Control	47 b	63 c	69 b	86 b
BA25	85 a	100 a	93 a	100 a
BA5	90 a	85 b	100 a	100 a
BA25IAA	85 a	100 a	98 a	100 a
BA5IAA	97 a	100 a	98 a	100 a
CV	1.66	1.22	1.23	0.75
Probability	0.001	0.0001	0.001	0.0001
R^2	0.19	0.22	0.18	0.11

Note. [¥]BA: 6-Benzyadenine, 25=0.25mg/L; 5=0.5mg/L, IAA: Indole-3-Acetic-Acid 0.01mg/L

Root induction was found to be dominated by a hormone-free medium in the Genovese variety as well as in the Red Rubin except for data collected on day 42 where the Red Rubin revealed no significant differences among treatments concluding that the addition of plant growth regulators in the multiplication stage, inhibited the formation of roots in most of the explants (Table 4). Furthermore, these results were found to be favorable for the stud given that an elevated growth of roots would detain shoot formation. Additionally, Asghari et al. (2012) and Gopi et al. (2006) evidenced that when supplementing IAA alone at low concentrations in their medium, there was a greater number of roots per explant in the rooting phase or stage III of micropropagation given that auxins are excellent PGRs for root proliferation.

Table 4

Effect of growth regulators on the root formation on days 21 and 42 at the multiplication stage of Genovese Basil and Red Rubin, percentage of explant with root formation

Treatment[¥] (mg/L)	Genovese		Red Rubin	
	Roots day 21 (%)	Roots day 42 (%)	Roots day 21 (%)	Roots day 42 (%)
Control	26 a	21 a	21 a	19
BA25	12 b	1 b	13 b	7
BA5	12 b	0 b	10 b	2
BA25IAA	10 b	1 b	10 b	2
BA5IAA	6 b	0 b	9 b	0
CV	2.23	1.57	2.49	0.69
Probability	0.0146	0.0001	0.0211	0.0683
R^2	0.04	0.34	0.04	0.03

Note. [¥]BA: 6-Benzyadenine, 25=0.25mg/L; 5=0.5mg/L, IAA: Indole-3-Acetic-Acid 0.01mg/L

Shoots and Leaves formation in Red Rubin Variety

The response of the treatments with BA along with the control were significantly superior compared to the treatments in combination with IAA when observing leaf and shoot development. The response of the development of leaves was best in the presence of BA on day 21, especially at the lowest concentration (0.25mg/L). However, results analyzed on day 42 showed that the effects of BA at both concentrations presented the same results as control treatment (Figures 4 and 5). These results are similar to Trettel et al. (2019) where they argue that at the end of the trial, the anatomical analysis of leaves of *O. basilicum* supplemented with the lowest concentration of BA (0.2mg/L) along with the control treatment, presented the highest averages of growth.

Moreover, the addition of IAA to the medium with BA did not stimulate growth on any variable (Table 5). The formation of shoots was delayed as the concentration of BA increased and IAA was added. According to Sharma et al. (2015) for the development of shoots, the dormancy of axillary buds must be broken, and this is obtained through the supplementation of the combination of both auxins and cytokinins at optimum concentrations. These results are consistent with Asghari et al. (2012) where it was reported that shoot regeneration and the average number of shoots formed decreased

as BAP concentration in the medium was elevated (2.5 mg/L) or combined with very low concentrations of IAA (0.06mg/L).

Table 5

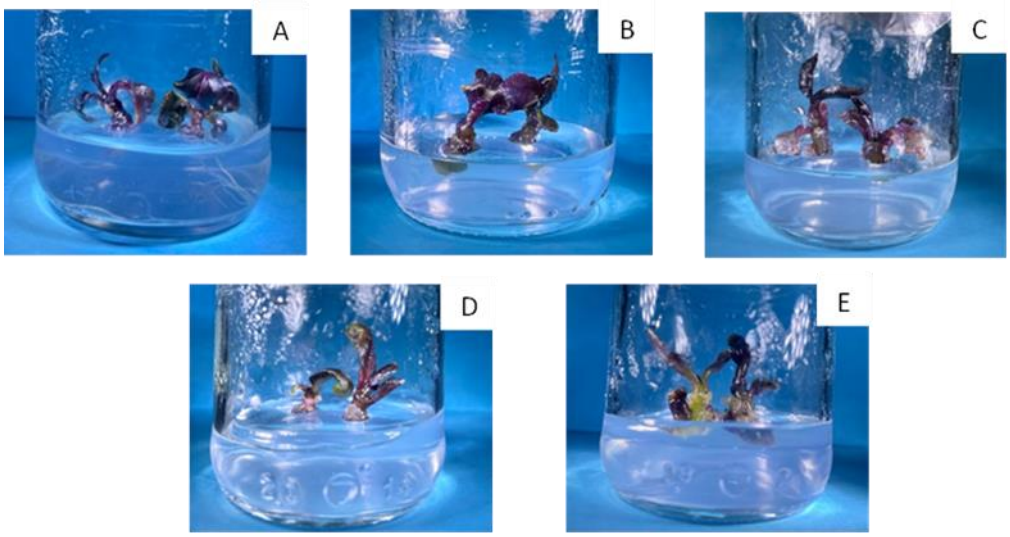
Effect of growth regulators on the number of leaves and shoots in in vitro multiplication of Red Rubin basil

Treatment[¥] (mg/L)	Red Rubin Basil			
	Leaves Day 21	Shoots Day 21	Leaves Day 21	Shoots Day 21
Control	2.38 bc	1.06 abc	2.34 a	1.31 a
BA25	3.07 a	1.13 a	2.10 a	1.32 a
BA5	2.70 ab	1.10 ab	2.13 a	1.30 a
BA25IAA	1.88 c	0.96 bc	1.61 b	1.06 b
BA5IAA	2.39 bc	0.98 bc	1.65 b	1.00 b
CV	5.78	1.38	4.21	1.72
Probability	0.0010	0.0160	0.0007	0.0001
R^2	0.06	0.04	0.07	0.11

Note. [¥]BA: 6-Benzyadenine, 25=0.25mg/L; 5=0.5mg/L, IAA: Indole-3-Acetic-Acid 0.01mg/L

Figure 4

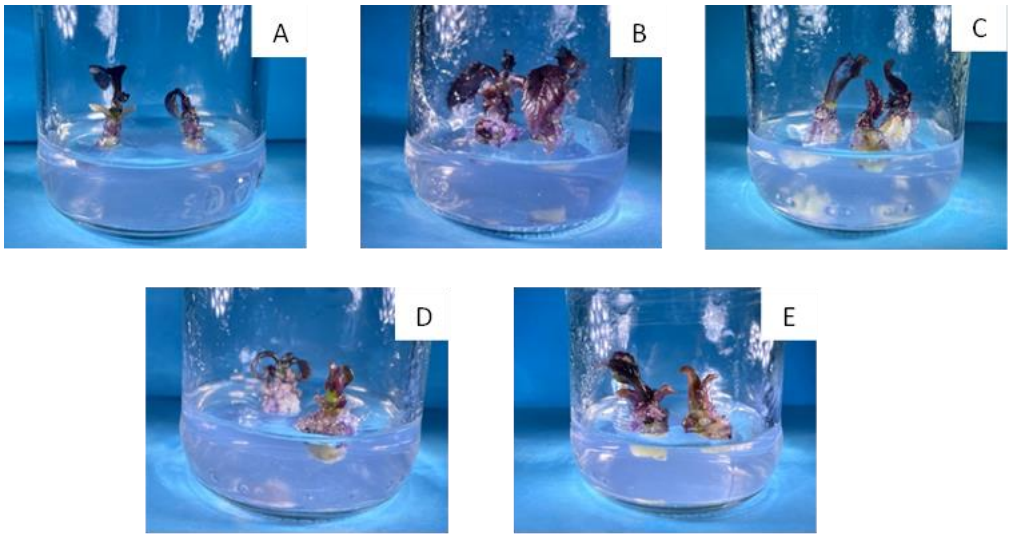
Effects of PGRs in Red Rubin Basil of in vitro multiplication on day 21



Note. (A) Control treatment (B) Treatment BA 0.25 mg/L (C) Treatment BA 0.5 mg/L (D) Treatment BA 0.25 mg/L and IAA 0.01 mg/L (E) Treatment BA 0.5 mg/L and IAA 0.01 mg/L.

Figure 5

Effects of PGRs in Red Rubin of in vitro multiplication on day 42



Note. (A) Control treatment (B) Treatment BA 0.25 mg/L (C) Treatment BA 0.5 mg/L (D) Treatment BA 0.25 mg/L and IAA 0.01 mg/L (E) Treatment BA 0.5 mg/L and IAA 0.01 mg/L.

Shoots and leaves formation in Genovese Basil Variety

The effect upon leaf growth in the Genovese variety is visible where BA was the most responsive at its growth rate. Both concentrations (0.25mg/L and 0.5mg/L) gave best results when analyzed at day 21 and day 42. The stimulatory effect of BA on leaf growth has been reported by Trettel et al. (2019) and Stefanova et al. (2011), concluding that the highest number of leaves was found in explants cultured in mediums with 0.2 mg/L and mediums in combination with auxins at low concentrations.

On day 21 of the Genovese variety, treatments show no significant difference between each other when observing shoot development (Figure 6), representing that with or without PGRs, shoot formation is prone to have a steady growth rate in the first weeks of culture, up until day 42 where differences were observed (Table 6).

Two weeks after the first data collection, the explants were subcultured and appeared to be stimulated by the BA supplemented. Results show a higher rate of shoot multiplication with BA, followed by the control treatment (Figure7). These results are similar to the reactions reported of the red rubin variety. Sharma et al. (2014) also reported success in achieveing a greater amount of shoots when BA was supplemented on its own in the medium, concluding that the integration of IAA decreased the number of shoots per explant than the ones with cytokinins alone. These findings differ from Siddique and Anis (2008) where the effects of BA in combination with IAA was the most responsive in the number of shoots developed per explant.

Table 6

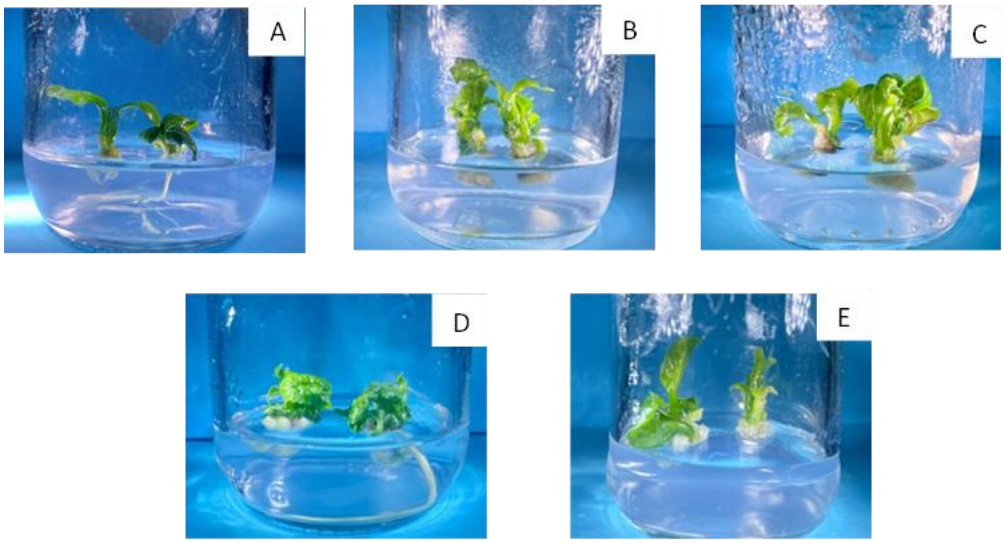
Effect of growth regulators on the number of leaves and shoots in in vitro multiplication of Genovese Basil

Treatment¥ (mg/L)	Genovese Basil			
	Leaves Day 21	Shoots Day 21	Leaves Day 21	Shoots Day 21
Control	2.64 c	1.13 a	3.14 c	1.16 c
BA25	4.10 ab	1.30 a	4.24 a	1.38 a
BA5	4.41 a	1.26 a	4.00 ab	1.30 a
BA25IAA	3.52 b	1.23 a	3.97 ab	1.00 c
BA5IAA	3.67 b	1.27 a	3.60 bc	1.00 c
CV	6.00	2.14	5.79	1.54
Probability	0.0001	0.4344	0.0036	0.0001
R^2	0.12	0.01	0.05	0.17

Note. ¥BA: 6-Benzyadenine, 25=0.25mg/L; 5=0.5mg/L, IAA: Indole-3-Acetic-Acid 0.01mg/L

Figure 6

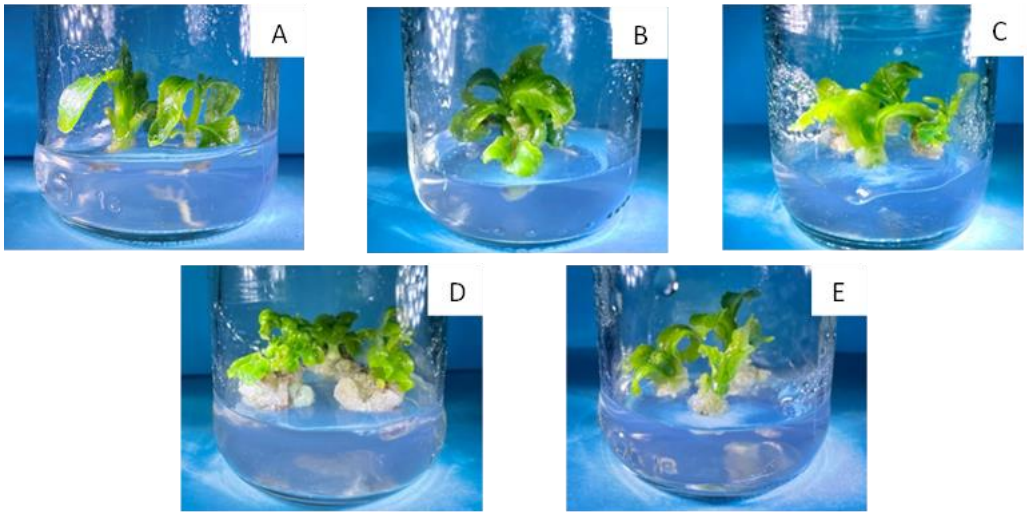
Effects of PGRs in Genovese Basil of in vitro multiplication on day 21



Note. (A) Control treatment (B) Treatment BA 0.25 mg/L (C) Treatment BA 0.5 mg/L (D) Treatment BA 0.25 mg/L and IAA 0.01 mg/L (E) Treatment BA 0.5 mg/L and IAA 0.01 mg/L.

Figure 7

Effects of PGRs in Genovese Basil of in vitro multiplication on day 42



Note. (A) Control treatment (B) Treatment BA 0.25 mg/L (C) Treatment BA 0.5 mg/L (D) Treatment BA 0.25 mg/L and IAA 0.01 mg/L (E) Treatment BA 0.5 mg/L and IAA 0.01 mg/L.

Conclusions

The treatments supplemented with BA were found to be the most effective to induce the highest number of leaves in both varieties.

In the Red Rubin variety, shoot multiplication would be better off in a plant growth regulator-free medium.

Genovese basil had significant effects with the treatment containing the lowest concentrations of BA in shoot formation after 42 days in multiplication.

Recommendations

Study the effects of increasing BA concentrations when passing the explants to the multiplication stage to promote the rate of shoot induction and leaf growth in both the Genovese and Red Rubin varieties.

Continue evaluating techniques to a successful subculture, such as passing shoots to a half strength medium to stimulate greater shoot proliferation.

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