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Optimizing in vitro regeneration and micropropagation of raspberry genotypes

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Abstract
This study investigates the in vitro regeneration and multiplication of three raspberry genotypes: RUVI, OPAL, and HERITAGE. Utilizing optimized Murashige-Skoog (MS) culture media enriched with varying concentrations of cytokinin BAP and gibberellin GA3, the research identifies hormonal variant V3 (0.5 mg/L BAP, 0.5 mg/L GA3) as the most effective for promoting shoot regeneration and multiplication. RUVI demonstrated the highest regeneration rate (60%) and superior rooting and acclimatization efficiencies (90% each), emphasizing its robustness and adaptability. HERITAGE showed moderate performance, while OPAL displayed the lowest rooting rate (60%), highlighting variability among genotypes. The study further underscores the critical role of acclimatization, where controlled environmental conditions ensured a smooth transition of regenerants from sterile in vitro setups to natural growth environments. These findings provide actionable insights for optimizing micropropagation techniques, enhancing the scalability and efficiency of raspberry cultivation programs.

Keywords: raspberry plants, in vitro culture, hormonal balance

Introduction

The raspberry is a shrub species known for its attractive, delicious, and highly nutritious fruits. A key advantage of raspberry plants is their early fruit-bearing capability and the potential for high yields. Raspberry fruits are consumed both fresh and processed in the food industry due to their exquisite taste and valuable chemical composition. These include sugars (5-7%), organic acids such as citric acid (1.0-1.5%), and significant amounts of vitamins, especially vitamin C (25-50 mg%), as well as essential minerals like potassium and calcium [6, 10]. Raspberry leaves, roots, and extracts are also utilized in traditional medicine for treating various ailments [5, 1, 12].

This study focuses on the in vitro regeneration and multiplication of three high-performing raspberry genotypes, aiming to produce uniform and healthy planting material. In vitro culture provides a rapid method for propagating valuable genotypes [2, 9]. The purpose of this technique is to generate large quantities of planting material within a short time frame, unaffected by weather conditions or seasonal changes. Additionally, in vitro methods rejuvenate biological material, overcoming challenges associated with conventional vegetative propagation, such as genetic degeneration and reduced resistance to environmental stresses [14].

Micropropagation, or in vitro multiplication, is a plant biotechnology technique that involves the use of in vitro cultures of cells, tissues, and organs for plant propagation [3]. Conducted in sterile, controlled laboratory conditions, micropropagation relies on artificial nutrient media containing water and essential nutrients for plant growth [7]. This method follows three main stages: initiation (sterilization and inoculation of plant fragments), multiplication (successive culture cycles to achieve the desired number of plants), and acclimatization (adaptation of plants to natural conditions in greenhouses and fields).

From an economic perspective, micropropagation is a promising technology, enabling the production of vast numbers of healthy plants efficiently [7]. It is particularly effective for species with low propagation rates through conventional methods [4, 11]. Through successive cycles, micropropagation can yield over a million plants from a single individual within a year, offering immense production potential. Additionally, in vitro material can be preserved for future use, ensuring availability during

climate challenges or natural disasters [11, 13]. Micropropagation also offers significant savings in land, energy, and labor compared to conventional methods, making it a highly efficient approach to large-scale plant multiplication.

Material and Method

For in vitro propagation, three raspberry varieties were selected: RUVI, OPAL, and HERITAGE.

RUVI is a summer-bearing raspberry variety of American origin. It forms tall and robust bushes with arched tips. The canes develop medium-to-long fruiting laterals that are well-anchored. This variety exhibits good tolerance to diseases and pests. The fruits are large, averaging 3.5 grams, conical-elongated in shape, bright red in color, and have a sweet-tart, aromatic flavor. The drupelets adhere well, ensuring resistance to transportation and handling. This variety is suitable for both fresh consumption and processing, being ideal for juices and freezing. RUVI matures at the end of June, making it a mid-season variety.

OPAL is a medium-vigor variety with upright canes. These fruit in their first year on approximately 1/3 to 1/2 of their length and have high suckering capacity. Under conditions of high atmospheric humidity, the fruits may be susceptible to gray mold. This variety demonstrates resistance to frost and drought. The fruits are conical in shape, bright red and glossy, with a pleasant taste and aroma. They are suitable for fresh consumption, freezing, and processing them into jams, juices, and jelly. Fruit maturation in the autumn season occurs between August and September, while in the summer season, it matures from late June to early July.

HERITAGE is a vigorous variety with thick, tall, and erect canes that are self-supporting. It fruits on 1/3 to 1/2 of the length of the growing canes. It is relatively cold-resistant and produces fruits of variable sizes depending on cane age and applied agro-technical practices. The fruits are firm and transport-resistant, dark red with a slightly dull appearance, and have a balanced, good taste but are not overly juicy. This variety is suitable for fresh consumption, freezing, and processing into jams and jellies. HERITAGE has two maturation periods: the summer harvest occurs in early July, while the autumn harvest extends from mid-August to the end of September.

An in vitro culture medium consists of an inorganic component (mineral salts containing macro- and microelements) and an organic component (carbohydrates, vitamins, amino acids, and growth regulators). Solid *in vitro* culture medium includes a gelling agent, such as agar. Water used for media preparation must be purified through deionization or distillation.

Media preparation involves using clean glassware, high-quality water, pure chemicals, and accurate measurements. The preparation process can include:

1. Using pre-packaged salt mixtures in powdered form.
2. Preparing concentrated stock solutions.
3. Weighing and dissolving individual salts according to the desired recipe.

Before sterilization, sucrose and thermostable hormones are added, and the pH is adjusted to 5.8. The sterilized medium is then poured into culture vessels under sterile conditions.

Sterilization Techniques

1. Glassware: Washed with detergent, rinsed with distilled water, and sterilized in dry heat at 180°C for 2-3 hours.
2. Plastic containers and materials: Autoclaved at 121°C for 15-20 minutes.
3. Work surfaces: Sterilized using UV light overnight or disinfectants.

Initiating in Vitro Cultures

Explants were sterilized through a series of steps:

1. Wash with tap water and detergent.
2. Immersion in 70% ethanol for 1 minute.
3. Immersion in 0.1% mercuric chloride solution for 2 minutes.
4. Rinsing five times with sterile distilled water.

Axillary buds were inoculated onto sterile culture media in a laminar airflow hood.

Multiplication via Axillary Budding

Axillary budding is crucial for commercial propagation as it determines multiplication rates. Steps include:

1. Initiating propagation with axillary bud fragments.
2. Stimulating multiple axillary shoots using cytokinin-enriched media
3. Harvesting shoots

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After inoculation, the sealed culture vessels were placed in the growth chamber, where they were incubated at a temperature of 24°C and exposed to light. Thirty days after inoculation, the resulting plantlets were used as explant sources for initiating in vitro culture. The buds selected for this process were derived from one-year-old shoots. During the regeneration and multiplication phase, the Murashige-Skoog (MS) basal medium (Murashige T., 1962) was used, enriched with different concentrations of hormones, including the cytokinin BAP (benzylaminopurine) and gibberellin GA3. Thus, three experimental variants were developed: V1, V2, and V3. Sucrose, added at a concentration of 30 g/L, served as the carbon source (Table 1).

Table 1. Hormonal balance used for regeneration from axillary buds

The explant used	***Culture medium***	***Hormonal balance***			***Environmental conditions***
Axillary buds	Murashige-Skoog *(MS)*	V_1 **1,5 mg/l BAP**	V_2 **1 mg/l BAP**	V_3 **0, 5mg/l BAP 0,5 mg/l GA_3**	24°C photoperiod of 16 h light, humidity 50-70%

Axillary Shoot Multiplication

This stage is critical for commercial propagation, as the multiplication coefficient is the primary economic criterion. The main objective of this phase is to obtain the highest possible number of usable shoots from each subculture, with dimensions appropriate for separation and as uniform as possible. The cycle of multiplication and harvesting generally lasts four weeks. Shoots developed in the previous stage are sectioned and transferred to a fresh rooting medium. To ensure efficient multiplication, each segment must contain at least one growth node.

In vitro micropropagation of raspberries was performed by stimulating the growth of axillary buds and promoting multiple axillary development, with each new shoot capable of becoming a potential plant. This technique aims to inhibit apical dominance and activate the meristems of existing buds on the plant, facilitated by the addition of cytokinins to the culture medium. Typically, nodes (axillary buds) are used as explants.

To continue the culture, nodal segments obtained from shoots grown in the previous culture were inoculated on MS medium enriched with the hormonal variant that demonstrated the most effective results for regeneration. Each plantlet produced 3 to 4 nodal segments, which were used to initiate new cultures.

Steps involved in axillary shoot multiplication:

1. Initiation of multiplication: Stem fragments containing at least one axillary bud, extracted from plantlets regenerated from mother plant buds, are used as the base material to start the multiplication process.
2. Stimulation of multiple axillary shoots: Dense clumps of shoots are induced through a culture medium enriched with cytokinin BAP and gibberellin GA3. This step promotes the multiple development of axillary shoots, leading to a higher density of plants.
3. Effective multiplication phase: Clumps of shoots are divided, and new multiplication cultures are initiated. This process is repeated to amplify the number of plants available for subsequent phases or direct planting.
4. Rooting process: Shoots can be rooted either in vitro or directly in soil. In vitro rooting allows better control over environmental conditions and ensures a higher success rate, while direct soil rooting is often used to acclimate shoots to external conditions, preparing them for final transplantation.

This multiplication method is highly efficient for the rapid and large-scale production of plants and is widely used in horticulture and agriculture for propagating valuable or difficult-to-multiply species.

In vitro rooting

The induction of rhizogenesis, or root formation, was achieved by cultivating the shoots on Murashige-Skoog (MS) medium without the addition of hormones. This process took place in the growth

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chamber, where the conditions were maintained at a constant temperature of 24 ^{0}C and a photoperiod of 16 hours of light followed by 8 hours of darkness.

This method did not guarantee rhizogenesis for all in vitro plantlets. Shoots that failed to develop roots under in vitro conditions were subsequently rooted during the acclimatization process.

Acclimatization of regenerants

The acclimatization of regenerants is a crucial step to ensure the survival of new plants under cultivation conditions. The transfer of plants generated in vitro to a septic environment is performed gradually through a stepwise acclimatization process.

One of the most critical factors during acclimatization is the limited water absorption capacity of the roots due to the still underdeveloped root system. Additionally, the foliar cuticle is poorly developed. The acclimatization period lasts between 4 and 6 weeks. This process must be gradual to allow plants to transition from in vitro to in vivo conditions. This includes adapting to lower relative humidity, developing mechanisms for stomatal opening and closing, and accelerating photosynthetic processes. During acclimatization, natural stimulation and gradual environmental changes aid in inducing rooting in shoots that initially failed to root in vitro, ensuring better survival and subsequent growth when plants are transplanted into the soil. Plants are irrigated with nutrient solutions.

Steps for the acclimatization process:

1. The plants are removed from the vessels where they were cultivated in vitro, and the culture medium is washed off the newly formed roots using running water.
2. The roots are trimmed (shortened) if they are excessively long.
3. The nutrient substrate, consisting of flower soil and perlite (in a 50% ratio), is moistened with water in preparation for planting.
4. Plants are introduced into the nutrient substrate, and the container is covered with a transparent foil.
5. Watering is done moderately, and the container lids are gradually opened\u2014initially for 2-3 minutes\u2014then exposure time to the ambient environment is progressively increased. After 3-4 weeks of planting, the plants are fully acclimatized.

Results and Discussions

The three genotypes studied responded differently regarding shoot regeneration, depending on the hormonal balance. The results are presented in table 2. According to the data, the highest regeneration rate was obtained in variant V3 for the Ruvi genotype.

Table 2. Results of shoot regeneration based on hormonal balance

GENOTYPE	NUMBER OF INOCULATE EXPLANTS	REGENERATION PERCENTAGE (%)		
		V1	***V2***	***V3***
RUVI	**25**	*9*	*22*	*60*
OPAL	**25**	*6*	*16*	*49*
HERITAGE	**25**	*7*	*19*	*53*

For the Ruvi genotype, the efficiency of the regeneration process varied significantly based on the hormonal medium used. In variant V3, the regeneration rate was 60%, demonstrating a considerably higher success rate compared to variants V1 and V2, where regeneration rates were 9% and 22%, respectively. This indicates a clear superiority of variant V3 in stimulating regeneration for this genotype (Figure 1).

For the other two genotypes, Opal and Heritage, better performance was also observed with the V3 hormonal variant, showing regeneration rates of 49% and 53%, respectively. These figures highlight the effectiveness of variant V3 in inducing regeneration for these genotypes, compared to the lower results obtained in V1 and V2 in all cases.

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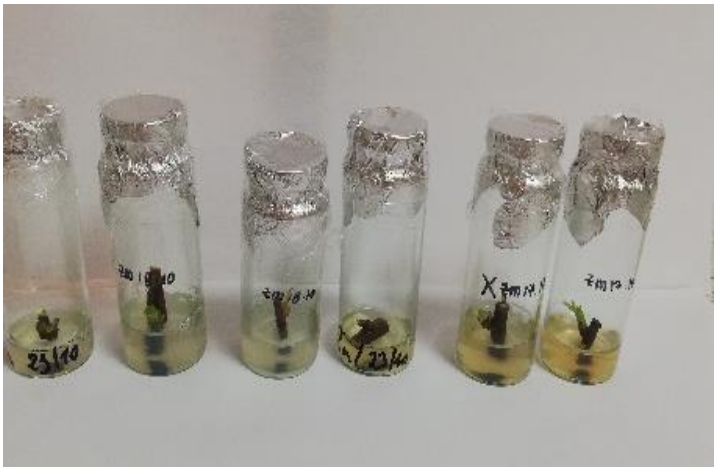

Figure 1. Regenerated shoots

These differences in regeneration rates can be attributed to the specific responses of each genotype to the different combinations of hormones present in culture media V1, V2, and V3. The results underline the importance of selecting the appropriate hormonal medium to optimize in vitro regeneration processes, tailored to the specificities of each plant genotype studied (figure 2). This bar chart illustrates the regeneration rates for the RUVI, OPAL, and HERITAGE genotypes across hormonal variants V1, V2, and V3.

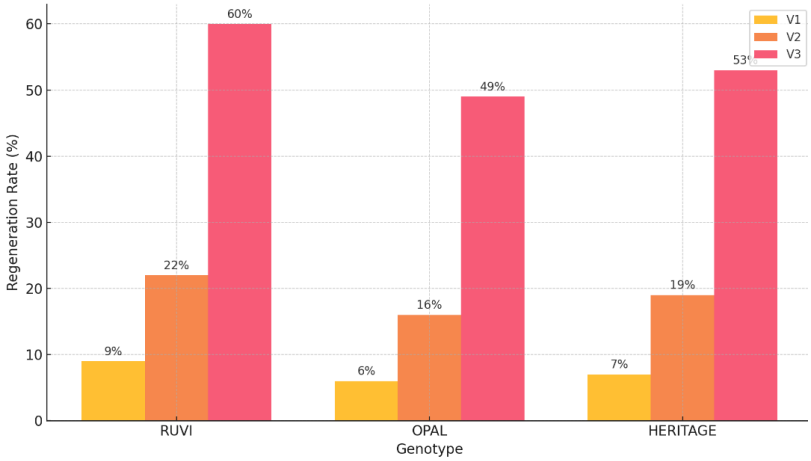

Figure 2. Influence of hormonal balance on meristem regeneration rate

Experimental results on axillary shoot multiplication – number and height of obtained shoots

The multiplication of regenerated shoots in the first cycle of aseptic cultivation was performed by subculturing them on the same basal medium used in the previous stage, using the hormonal balance that proved to be the most efficient for regeneration. This was the MS medium supplemented with 0.5 mg/L BAP (benzylaminopurine) and 0.5 mg/L GA3 (gibberellin), referred to as variant V3, which was the most effective in stimulating both regeneration and multiplication.

In vitro shoots began to appear 10 days after the culture initiation, an early indication of the success of the multiplication procedure through axillary shoot proliferation. Detailed monitoring of shoot development was performed by counting shoots and measuring their height at 6 and 8 weeks after culture initiation. These evaluations provided valuable insights into shoot growth and development, highlighting the effectiveness of the hormonal medium used (Figures 3 and 4).

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Figure 3. Regenerated shoots after 4 weeks

Figure 4. Regenerated shoots after 8 weeks

Through this subculturing method, regenerated shoots continued to grow under optimal conditions, ensuring efficient and sustainable production of new plants. This is a common practice in vitro culture technique, allowing rapid multiplication of agriculturally or scientifically valuable plants, while ensuring uniformity and quality of the plant material obtained (table 3 and figure 5).

Table 3. Number of regenerated shoots

Genotype	Hormonal balance	6 weeks		8 weeks	
		x± sx	***S %***	***x± sx***	***S%***
RUVI	V3	7,28±3,54	48,64	9,16±2,44	26,68
	V2	6,44±2,94	45,73	8,08±2,23	27,65
	V1	5,48±2,00	36,54	6,68±1,40	21,04
OPAL	V3	6,76±1,98	29,36	8,28±3,11	37,63
	V2	5,92±1,44	29,31	6,68±1,40	21,04
	V1	4,92±1,44	29,28	5,4±2,43	45,04
HERITAGE	V3	6,68±1,62	24,33	8,16±2,98	36,53
	V2	5,88±2,45	41,75	6,72±1,79	26,66
	V1	4,84±1,43	29,63	5,36±1,55	28,94

Note: Results are expressed as mean ± standard deviation

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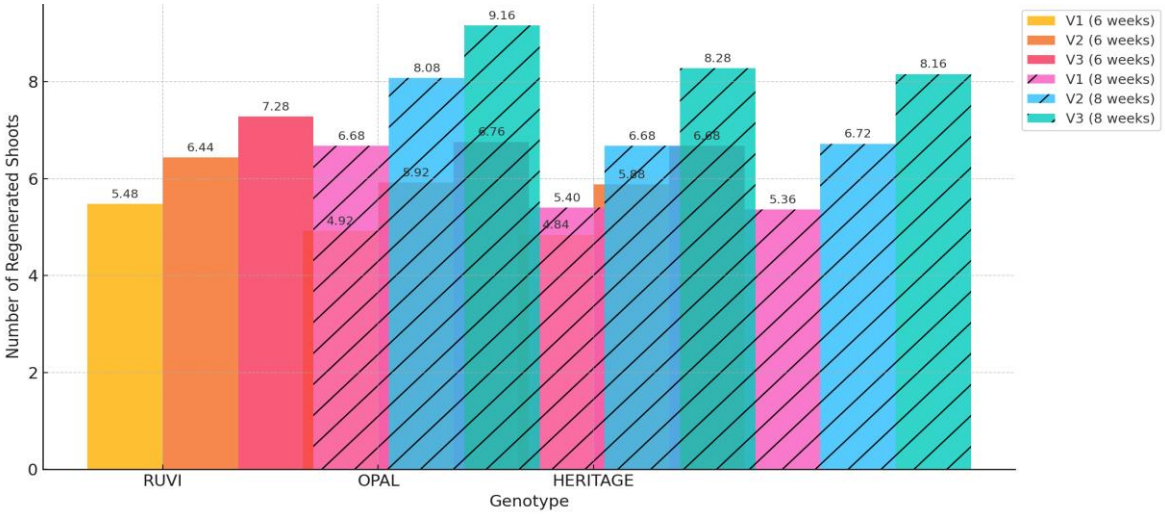

Figure 5. Number of regenerated shoots by genotype and hormonal balance (6 and 8 weeks)

The graph depicts the number of regenerated shoots for the genotypes RUVI, OPAL, and HERITAGE across three hormonal variants (V1, V2, and V3) over two-time intervals: 6 weeks and 8 weeks.

After 6 weeks RUVI consistently shows the highest number of regenerated shoots across all hormonal variants, with V3 being the most effective (7.28). OPAL and HERITAGE follow similar patterns but with slightly lower shoot numbers.

After 8 weeks the number of shoots increases significantly for all genotypes. RUVI continues to perform best with V3 (9.16 shoots), while OPAL and HERITAGE also achieve substantial growth with V3.

Table 4. Average shoot height

Genotype	Hormonal balance	6 weeks		8 weeks	
		x± sx	***S %***	***x± sx***	***S%***
RUVI	V3	3,14±1,29	41,79	4,44±0,71	16,03
	V2	2,76±1,12	40,88	4,28±0,84	19,68
	V1	2,52±0,87	34,59	3,72±0,89	23,94
OPAL	V3	2,8±0,76	27,27	3,96±0,84	21,22
	V2	2,68±0,74	27,92	3,56±0,82	23,04
	V1	2,48±0,50	20,56	3,28±0,61	18,71
HERITAGE	V3	2,72±0,54	19,91	3,8±0,70	18,60
	V2	2,64±0,48	18,55	3,44±0,71	20,69
	V1	2,4±0,57	24,05	3,16±0,74	23,61

Note: Results are expressed as mean ± standard deviation

The graph illustrates the average shoot heights for the genotypes RUVI, OPAL, and HERITAGE across two-time intervals: 6 weeks and 8 weeks. Each hormonal balance (V1, V2, V3) is represented for both time points.

After 6 weeks RUVI shows the highest average shoot height across all variants, particularly with V3 (3.14 cm), followed by OPAL and HERITAGE.

After 8 weeks, the average shoot heights increased for all genotypes, with RUVI achieving the greatest height (4.44 cm with V3). OPAL and HERITAGE also exhibited significant growth, highlighting the effectiveness of the hormonal treatments over time.

The graph demonstrates that the hormonal balance significantly impacts the growth rates, with V3 consistently yielding better results across all genotypes.

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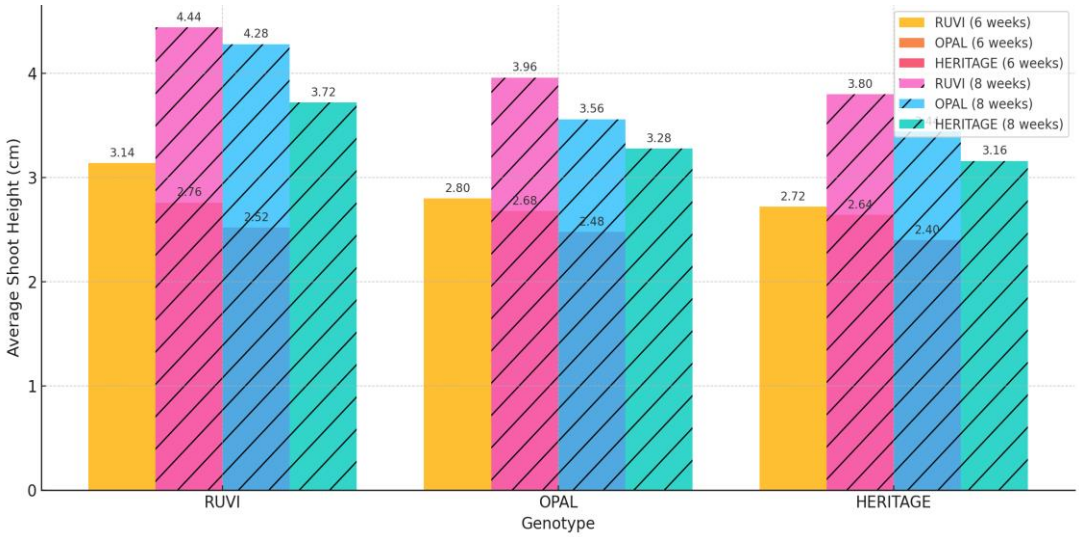

Figure 6. Average Shoot Height by Hormonal Balance (6 and 8 weeks)

Experimental results on rooting and acclimatization of raspberry regenerants

Shoot elongation and rooting were achieved through aseptic subculturing on the same MS medium supplemented with 0.5 mg/L GA3 (Table 5).

Figure 7. In vitro rooted shoots

Table 5. Results on shoot rooting and acclimatization

Genotype	No. of inoculated shoots	Rooting in vitro		Acclimatization		
		No. of rooted shoots	Rooting rate in vitro	No. of transferred shoots	No. of acclimatized shoots	Acclimation rate
Ruvi	15	13	90%	15	13	90%
Opal	15	10	60%	15	12	80%
Heritage	15	12	80%	15	13	90%

The Ruvi genotype stood out for its highest rooting and acclimatization rates compared to the other two genotypes studied. During acclimatization, the plants were transferred to plastic trays containing a nutrient substrate suitable for plant growth. The trays were equipped with lids to maintain a humid and controlled environment, essential for successful acclimatization (figures 7 and table 5).

During the acclimatization phase, shoots that did not develop roots in vitro were able to root due to controlled humidity and temperature conditions in the growth chamber, which mimicked a natural environment with reduced stress. This stage is essential as it ensures the transition of plants from the sterile and controlled laboratory conditions to a more variable and less controlled environment, preparing the plants for subsequent growth in the field or other external environments.

Conclusions

Based on the results obtained, the following conclusions can be drawn:

1. Key conclusion regarding the multiplication procedure of regenerated shoots in the first cycle of aseptic cultivation: The use of an optimized hormonal medium, such as variant V3 with the addition of 0.5 mg/L BAP and 0.5 mg/L GA3 in the MS culture medium, was decisive for the success of regeneration and multiplication. This approach not only promoted the appearance of in vitro shoots within a relatively short time of 10 days from initiation but also supported the robust development of shoots, as reflected in the measurements taken at 6 and 8 weeks.
2. Conclusions regarding the rooting and acclimatization rates of the Ruvi, Opal, and Heritage genotypes:

a. High efficiency for the Ruvi genotype: The Ruvi genotype demonstrated superior adaptability and survival capacity, achieving the highest rooting and acclimatization rates (90% for both processes). This indicates particular robustness of this genotype, suggesting it is an excellent choice for cultivation under similar laboratory conditions and potentially in the field due to its stability.

b. Variability in the results of the Opal and Heritage genotypes: Although both genotypes showed good acclimatization rates (80% for Opal and 90% for Heritage), their in-vitro rooting rates varied (60% for Opal and 80% for Heritage). This suggests a different sensitivity to the initial rooting process, likely influenced by genetic differences between the two types. Heritage appears to be more adaptable than Opal, with performance closer to that of Ruvi.

c. Importance of the acclimatization process: All genotypes exhibited relatively high acclimatization rates compared to their in vitro rooting rates, indicating that the acclimatization process was well-managed and that the growth chamber conditions were suitable to support plant survival during this critical phase.

These conclusions provide valuable insights for optimizing micropropagation procedures and improving the efficiency of in vitro cultures, contributing to the overall success of plant multiplication and acclimatization programs.

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