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Effect of Growth Regulator Combinations on *In Vitro* Shoot Proliferation and Root Formation in *Fragaria vesca* and *Fragaria × ananassa*

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Abstract
The present study aimed to evaluate the influence of hormonal balance on *in vitro* multiplication and rooting in two *Fragaria* species (*Fragaria vesca* and *Fragaria* × *ananassa*). The biological material consisted of plants derived from seeds, cultured on Murashige and Skoog (MS) medium supplemented with various combinations of growth regulators. Four hormonal variants were tested for the multiplication stage: V1 – BAP 2 mg/L + IAA 0.5 mg/L; V2 – BAP 4 mg/L + IAA 0.5 mg/L; V3 – TDZ 1 mg/L + IAA 0.5 mg/L; and V4 – TDZ 2 mg/L + IAA 0.5 mg/L. For the rooting stage, shoots were transferred onto MS media with the following variants: R1 – MS without hormones; R2 – MS + NAA 1 mg/L; and R3 – MS + NAA 2 mg/L.
The results revealed significant differences between the two species regarding shoot proliferation rate and root formation, depending on the type and concentration of growth regulators. In general, TDZ-containing media induced higher shoot multiplication, particularly in *Fragaria* × *ananassa*, whereas NAA-supplemented media promoted root initiation, especially in *Fragaria vesca*. These findings highlight the importance of optimizing the hormonal balance to develop efficient micropropagation protocols for *Fragaria* species.

Keywords: *in vitro* culture, micropropagation, culture media, hormonal balance

Introduction

In vitro culture techniques represent essential tools for the rapid and controlled propagation of plants, especially for horticultural species of high economic importance. Among these, the genus *Fragaria* (strawberry) holds a prominent place due to its nutritional, economic, and pharmaceutical value. Conventional propagation methods based on stolons are often limited by their slow multiplication rate, susceptibility to pathogen transmission, and dependence on environmental factors [1]. In this context, micropropagation provides an effective alternative for obtaining uniform, disease-free plant material available throughout the year [2,3].

The composition of the culture medium, particularly the hormonal balance between cytokinins and auxins, plays a decisive role in determining the success of *in vitro* multiplication. Cytokinins such as 6-benzylaminopurine (BAP) and thidiazuron (TDZ) are responsible for the initiation and proliferation of adventitious shoots, whereas auxins such as indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) influence elongation and rooting processes [4–6].

The classical work of Murashige and Skoog (1962) established the standard nutrient composition of the MS medium, which remains the basis for most plant tissue culture protocols [7]. Subsequent studies have shown that the effect of growth regulators varies depending on species, genotype, explant physiological state, and culture conditions [8,9]. The general principles of *in vitro* plant cultivation and the mechanisms by which growth regulators influence morphogenesis have been extensively described by George and collaborators [4,20], who emphasized the critical importance of hormonal balance and environmental parameters in determining morphogenetic response.

In *Fragaria vesca* and *Fragaria* × *ananassa*, significant differences have been reported in response to hormonal treatments. *F. vesca* is characterized by a faster germination rate and more vigorous shoot development, whereas *F.* × *ananassa* tends to produce a higher number of adventitious shoots of smaller size [10,11]. Debnath (2009) demonstrated the effectiveness of TDZ–IAA combinations in stimulating *Fragaria*

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shoot proliferation, while Litwińczuk et al. (2016) observed favorable effects of BAP at moderate concentrations on direct regeneration [12,13].

The aim of the present study was to evaluate the influence of hormonal balance on *in vitro* multiplication and rooting in two *Fragaria* species by testing four hormonal combinations for the multiplication stage and three variants for rooting. The final objective was to identify the optimal culture medium that ensures a high shoot proliferation rate and efficient root system formation, with practical applicability in large-scale propagation and breeding programs.

Materials and Methods

Plant material

The biological material used in the experiment consisted of seeds of two *Fragaria* species, *Fragaria vesca* and *Fragaria* × *ananassa*, purchased from a certified commercial supplier. All experiments were conducted under aseptic conditions to ensure contamination-free cultures.

Seed sterilization and germination

Seeds were first rinsed with tap water and soaked for 24 hours to promote imbibition. Under a laminar airflow cabinet, surface sterilization was performed using 0.1% mercuric chloride ($HgCl_2$) solution for 1.5 minutes, followed by three rinses with sterile distilled water. The sterilized seeds were inoculated onto Murashige and Skoog (MS) medium solidified with 6.5 g/L agar and supplemented with 3% sucrose as a carbon source. The pH of the medium was adjusted to 5.8 prior to autoclaving (121°C, 25 min). Cultures were incubated at 25 ± 2°C under a 16 h light/8 h dark photoperiod. Seeds were germinated under a 16 h light / 8 h dark photoperiod, which is commonly used for *in vitro* germination of strawberry species because long-day conditions enhance photosynthetic activation and promote uniform and rapid radicle emergence, as previously reported in Fragaria seed culture studies [21; 22]. Germination percentage was recorded after 4 and 7 days of incubation.

Shoot multiplication

For the multiplication stage, four hormonal variants were tested on MS medium:

- **V1:** BAP 2 mg/L + IAA 0.5 mg/L
- **V2:** BAP 4 mg/L + IAA 0.5 mg/L
- **V3:** TDZ 1 mg/L + IAA 0.5 mg/L
- **V4:** TDZ 2 mg/L + IAA 0.5 mg/L

Cultures were maintained under identical incubation conditions. Observations were made at 14 and 28 days after inoculation, and the mean number of adventitious shoots and mean shoot length were recorded.

Root induction

Elongated shoots were transferred to MS rooting media containing three hormonal variants:

- **R1:** MS without hormones
- **R2:** MS + NAA 1 mg/L
- **R3:** MS + NAA 2 mg/L

The cultures were maintained for 25 days, and the average number and length of roots per plantlet were measured.

Data analysis

Results were expressed as arithmetic means, and comparisons among treatments were based on relative differences and observed trends. Data interpretation was supported by previous findings on the response of *Fragaria* species to similar growth regulator combinations [14,15].

Results and Discussion

Aseptic seed germination

The results indicated a high germination capacity for both studied species, with distinct differences between *Fragaria vesca* and *Fragaria* × *ananassa*. After 4 days of incubation, the germination rate reached 60% in *F. vesca* and 45% in *F.* × *ananassa*, while after 7 days it increased to 99% and 81%, respectively (Table 1).

Table 1. Seed germination rate at 4 and 7 days after inoculation

No.	Genotype	Day 4 (%)	Day 7 (%)
1	*Fragaria vesca*	60	99
2	*Fragaria x ananassa*	45	81

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This variation can be explained by genotypic and physiological differences between the two species, as *F. vesca* is diploid and generally exhibits a more stable metabolism and uniform response under *in vitro* conditions [16]. Similar observations were made by Popescu et al. (2020), who reported faster germination in diploid strawberry forms compared to octoploid hybrids [17].

The almost complete germination recorded after one week confirms the efficiency of the sterilization procedure and the suitability of the MS medium composition. According to Boxus (1974), an MS medium with pH 5.8 and 3% sucrose content promotes early metabolic activity of embryos and enhances radicle elongation [18].

***In vitro* shoot proliferation – adventitious bud formation**

Adventitious buds were directly induced on the cultured explants, following a direct organogenesis pathway influenced by the type and concentration of growth regulators. After 14 days, clear differences among the hormonal variants were observed, and after 28 days, both species showed a pronounced increase in shoot number (Figure 1).

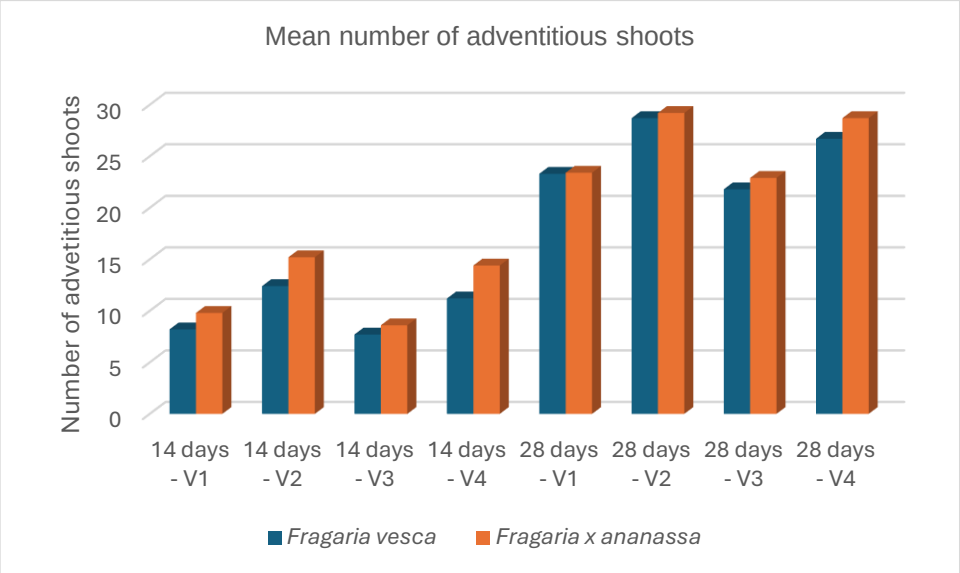

Figure 1. Mean number of adventitious buds formed at different time intervals on four hormonal variants in two *Fragaria* species

In general, BAP-containing variants induced a higher number of adventitious buds compared to TDZ-containing ones. In *Fragaria* × *ananassa*, a stronger multiplication capacity was observed during the first 14 days, particularly on variant V2 (BAP 4 mg/L + IAA 0.5 mg/L), which yielded an average of 15.2 shoots per explant, compared to 12.4 in *F. vesca*. After 28 days, values became more similar, indicating stabilization of proliferation dynamics.

These results agree with those of Debnath (2009), who highlighted the beneficial effect of BAP in stimulating shoot initiation in *Fragaria*, while noting that TDZ, although highly active, can cause hyperhydricity and reduced shoot quality [12]. Kulus et al. (2019) also reported that TDZ concentrations above 1 mg/L often lead to excessive water accumulation in tissues, reducing plantlet vigor [19].

In the present study, variants V3 (TDZ 1 mg/L + IAA 0.5 mg/L) and V4 (TDZ 2 mg/L + IAA 0.5 mg/L) induced fewer buds, but these showed a compact morphology and intense green color, suggesting good photosynthetic activity. Conversely, BAP treatments produced more numerous but slightly etiolated shoots, consistent with the findings of Litwińczuk et al. (2016) for *Fragaria* × *ananassa Duch.* [13].

Comparatively, *F. vesca* generated fewer buds but with more vigorous elongation and robust appearance. These interspecific differences likely reflect distinct hormonal sensitivities and metabolic responses, as previously reported by Rahman et al. (2018) for *Fragaria indica* [20].

Shoot elongation and plantlet development

After subculturing the adventitious buds onto hormone-free MS medium, a significant increase in shoot elongation was observed. Twenty days after transfer, the mean length of plantlets originating from the BAP 4 mg/L (V2) and TDZ 2 mg/L (V4) variants was the highest, ranging between 25–29 mm in *F. vesca* and 24–25 mm in *F.* × *ananassa* (Figure2).

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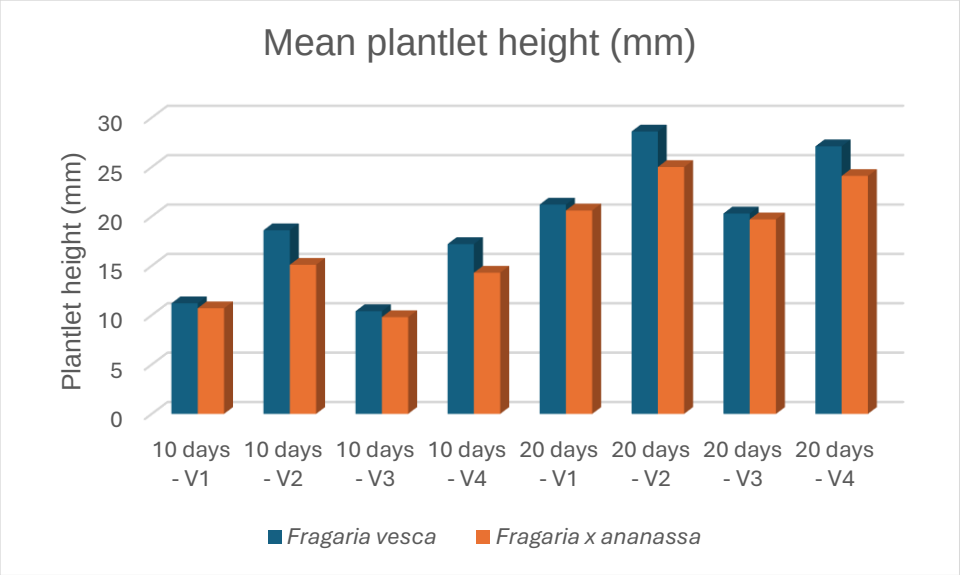

Figure 2. Mean plantlet height (mm) from four hormonal variants of the multiplication medium in two *Fragaria* species

These results confirm the positive role of the elongation stage on hormone-free medium, during which plants resume their normal physiological growth. Bornman et al. (2011) noted that the absence of cytokinins during this phase restores the natural auxin–cytokinin ratio, leading to improved internode elongation [21].

In this study, *F. vesca* showed superior elongation, even though it produced fewer buds during the proliferation phase. This observation aligns with the findings of Silva et al. (2022), who reported that diploid strawberry genotypes often compensate for lower bud numbers by displaying accelerated vegetative growth [22].

***In vitro* rooting**

Root induction was carried out on MS medium with three hormonal variants (R1 – hormone-free; R2 – NAA 1 mg/L; R3 – NAA 2 mg/L). After 25 days, both species formed roots under all treatments, regardless of auxin presence. However, the mean number of roots was slightly higher in R2, reaching 9.8 roots per plantlet for *F. × ananassa* and 9.0 for *F. vesca*. Doubling the NAA concentration (R3) did not further increase root number, indicating that higher auxin levels can exert inhibitory effects [23] (Figure3).

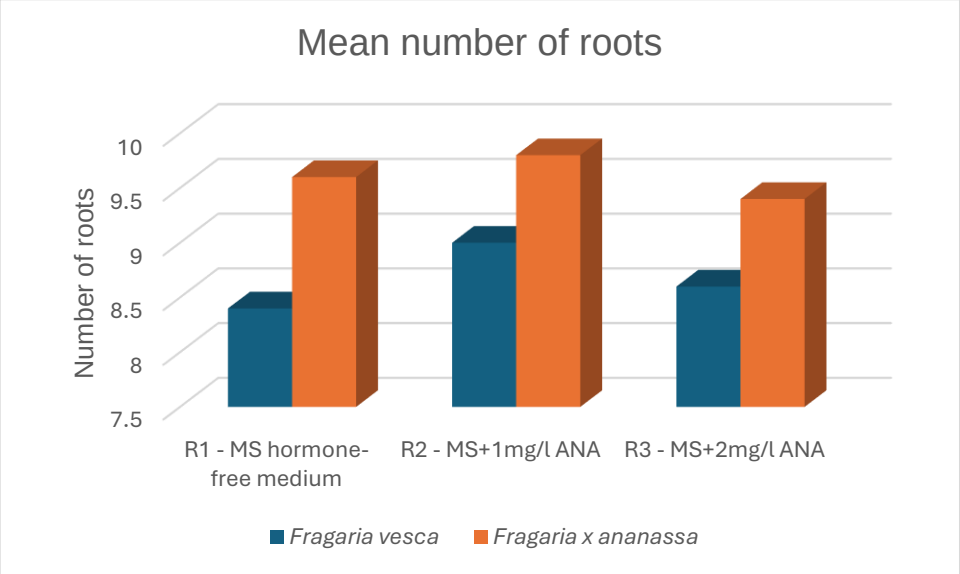

Figure 3. Mean number of roots formed in two *Fragaria* species on three hormonal variants

These findings are consistent with those of Singh et al. (2017), who showed that moderate NAA concentrations (0.5–1.0 mg/L) are optimal for root formation without compromising plantlet vigor [24]. Similarly, Chae and Lee (2015) observed efficient rooting of *Fragaria* × *ananassa* even on hormone-free media, likely due to residual endogenous auxins accumulated in previous culture stages [25].

Root length varied between 25–29 mm, with slightly higher values in *F. vesca*, suggesting a balanced relationship between root number and elongation. Morphologically, roots were fine, white, and highly branched — typical features of healthy *in vitro* plantlets.

Overall, the data confirm that efficient rooting can be achieved even on hormone-free MS medium (R1), which reduces production costs and simplifies the micropropagation protocol. Similar conclusions were reported by Pop et al. (2021), who recommended the use of MS0 medium for final regeneration of strawberry plantlets [26].

Conclusions

The present study demonstrated the significant influence of hormonal balance on the *in vitro* multiplication and rooting of two *Fragaria* species—*Fragaria vesca* and *Fragaria* × *ananassa*. The experimental findings led to the following main conclusions:

1. **Germination capacity** was higher in *F. vesca* (99%) than in *F.* × *ananassa* (81%), indicating a superior adaptability of the diploid species to *in vitro* culture conditions.
2. **Shoot proliferation** was strongly affected by the type and concentration of growth regulators. BAP-based variants produced more adventitious buds than TDZ-based ones, although the shoots were more elongated and delicate.
3. **Thidiazuron (TDZ)**, while less effective in producing large numbers of buds, generated compact, intensively green shoots—features desirable for maintaining physiological quality.
4. **The elongation phase** on hormone-free MS medium yielded vigorous plantlets, confirming the importance of reducing exogenous hormonal pressure in the later stages of micropropagation.
5. **Root induction** occurred successfully on both auxin-supplemented and hormone-free media, suggesting that rooting can be achieved efficiently without additional growth regulators, thus lowering production costs.
6. **The optimal hormonal combination** for multiplication was BAP 4 mg/L + IAA 0.5 mg/L, while the best rooting performance was obtained on hormone-free MS medium.
7. The results contribute to the development of standardized micropropagation protocols for *Fragaria* species, useful for breeding, germplasm conservation, and large-scale plant production.

Overall, this research highlights the critical role of hormonal equilibrium in determining the efficiency of regeneration processes and emphasizes species-specific responses within the genus *Fragaria*. Further studies involving additional growth-regulator combinations and ex vitro acclimatization performance will help refine and validate optimized micropropagation technologies for commercial use.

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