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Micropropagation of Arugula Plant (*Eruca sativa* Mill.)

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Abstract
Tissue culture, an essential technique in modern plant biology, offers promising avenues for the mass production of elite plant varieties for desirable traits. This study compared the effects of 0.1 mg L^{-1} NAA- 0.25 mg L^{-1} BAP plant growth regulator combination using Cotyledon leaves (0.4 cm^2) of three *Eruca* cultivars Estht 195, Estht 198, and Estht 201 of arugula (*Eruca sativa* Mill.) cultured for four and six weeks. Significant differences were noted among them for callus induction percentage, shoot induction percentage, and plant height (cm). Notably, var. Estht 201 indicated consistently improved performance, with a callus formation percentage of 61.83 % in 4 weeks and 74.05 % in 6 weeks. Similarly, Var. Estht 201 exhibited higher shoot induction (Week 4 67.45 %, Week 6:69.9 %), and plant height (Week 4:7.50 cm, Week 6:9.6 cm), throughout the experiment. These findings contributed to a deeper understanding of arugula micropropagation Dynamics. At the same time, this study has once again shown how important tissue culture micropropagation protocols are in terms of improving yield and quality as well as breeding studies.

Research Article

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1. Introduction

Arugula (*Eruca sativa* Mill.) is an edible annual plant in the family Brassicaceae utilized as a leaf vegetable for its distinguished, tart, and severe peppery flavor. It is called nursery rocket or Eruca in England, Australia, South Africa, Ireland, New Zealand and roka in Turkey.

E. sativa is popular for making mixed greens vegetable and salad in the Mediterranean region of Turkey, and is utilized as nourishment for its sharp flavor and bountiful supplements (potassium, sulfur, iron, and nutrients A and C) in leaves (Freitas et al., 2017; Nascimento et al., 2022). Arugula is spread in the Central and East Mediterranean region, (Barazani et al., 2012). It can be used in pharmaceutics, and spice industries, and as fresh vegetables (Hadi et al., 2017). Arugula has additionally been utilized for most restorative purposes as its leaves contain pharmaceutically important compounds, which are rich in glucosinolates and cell reinforcements with demonstrated anti malignant properties (Traka, 2016; Wilson et al., 2017; Tripodi et al., 2017; Jaafar and Jaafar, 2019; Nascimento et al., 2022). It is most often confused with *Diplotaxis tenuifolia*, known as" perennial wild rocket" from another species of the same family (Brassicaceae) that is utilized similarly (Reis et al., 2022; Banjac et al., 2023).

Cultivation of arugula negatively affects multiplication of pests and plant diseases (Srivastava et al., 2020; Panić et al., 2021; Kular and Kumar, 2011; Soroka et al., 2013). Hybridization with Brassica species may improve them for qualitative traits. Tissue culture can aid rapid breeding. Banjac et al. (2023) reported that, different tissue culture studies performed on the arugula are complex and responses to growth regulators vary.

No single medium is recommended as being completely agreeable for multiplication of a wide range of plant tissues and organs. Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S) are inorganic components expected by cells in larger amounts. The fundamental micronutrients expected by plant cells incorporate iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). Chelated forms of iron and zinc are frequently utilized in several culures (Anonymous, 2024).

Phytohormones have different effects in tissue culture studies. Cytokinins are knowm to invigorate cell division, prompt shoot arrangement, axillary shoot multiplication and impede root development (Anonymous, 2024).

The employment of auxin and cytokinin together is thought as the most crucial component for regulating growth and regeneration (Küplemez and Yıldırım, 2020). The auxins primarily affect root induction (Khawar et al., 2004).

In plant tissue and organ cultures, cytokinins, auxins, and cytokinin-auxin relationships are generally considered the most important factors in regulating growth and initiating regeneration (George, 2008). The relationship between cytokinin and auxin is important for the formation of the entire plant body because they play crucial roles in controlling many aspects of developmental processes and the establishment of meristems (Su et al., 2011; Küplemez and Yıldırım, 2020).

The study aimed to select disease-free arugula, through callus culture and distinguish the best genotype for inducing callus under in vitro conditions.

2. Materials and Methods

This study was carried out at Uşak University in 2023. Arugula seeds were obtained from the Molecular Biology and Genetics Department of the same university. The seeds were sown in pots at a depth of 1 cm, filled with field soil, followed by seeds germination. Thereafter, the cotyledon leaves (0.4 cm^2) were used for micropropagation of three species mentioned above.

Preparation of stock solution: The stock solutions of MS medium (Murashige and Skoog, 1962) were prepared for vitamins+ amino acids, Fe EDTA, major and

micronutrients, separately by weighing their respective amounts and dissolving them in one liter of distilled water. They were stored at 4 °C for 6 months.

NAA, and BAP were prepared by weighing 10 mg each dissolving them in 1-2 drops of ethanol or 1 N NaOH followed by adding water to complete the volume to 10 ml. They were stored at 4 °C for 6 months.

Preparation of cultures: The cultures consisted of the following components: MS medium, 30 g l^{-1} sucrose as carbon source, desired amounts of 0.1 mg L^{-1} NAA, and 0.25 mg L^{-1} BAP used as plant growth regulators, and 3.5 g l^{-1} phytagel as solidifying agent.

Double distilled water was used to prepare each culture. The pH of the solution was adjusted to 5.6±0.1 with 1N NaOH or 1N HCl solution at room temperature before adding Phytagel. The electrodes of pH meter were rinsed with distilled water before use and after each use to eliminate cross-contamination. These were covered and labeled accordingly and then autoclaved for 20 min at 121 °C and 4.15 kilopascal pressure.

The explants were surface sterilized using 1 % bleach diluted with sterilized distilled water. The forceps, tissue papers, petri dishes, and bottles were dry sterilized at 160 °C for 2 hours. The whole laboratory and the laminar flow cabin were also disinfected using 96 % ethanol.

The plants were passed through running tap water for 2-3 minutes followed by rinsing with double distilled water. The explants were surface disinfected using 20 % commercial bleach for 10 min, followed by rinsing them for 7×10 min with sterile distilled water.

Culture and growth room conditions: All cultures were kept in a growth room programmed to continuously provide 16 h light and 8 hours darkness cycle, using light intensity of 3000 Lux, and temperature of 24 °C± 2ºC throughout the experiment.

2.1. Statistical analysis

The statistical analysis (One way ANOVA) was performed using SPSS 26. The means were compared using LSD test. All values given in percentage were arcsine transformed before analysis.

3. Results and Discussion

3.1. Effect of blends of 0.1 mg L^{-1} NAA-0.25 mg L^{-1} BAP on regeneration from variety Estht 195, Estht 198, and Estht 201 after 4 weeks of culture

Treatments showed significant differences among means of callus formation and shoot induction percentage at 0.01 level, plant height and number of leaves per explant at 0.05 level. Callus induction started after 2 weeks of culture and terminated after 3 weeks to induce shoots. These were counted after 4 weeks of culture.

The results of weeks 4 analysis elucidate the complex interplay between hormonal treatments and arugula variety Estht 201 (Figure 1) in micropropagation. Variations in callus formation, leaf production, plant height, and shoot development underscore the genetic diversity among arugula varieties. Moreover, the significant influence of hormonal treatments highlights need of optimizing the tissue culture protocols for enhanced arugula regeneration.

This analysis provides insights into the performance of different arugula varieties under the effects of hormonal treatments, elucidating key factors influencing arugula micropropagation success.

The means of significant parameters after Duncan multiple range test are shown in Table 1. Which explains the effects of 0.1 mg L^{-1} NAA-0.25 mg L^{-1} BAP treatments on three arugula varieties after 4 weeks of tissue culture, Variety Estht 201 induced maximum callus formation (61.83 %), followed by Variety Estht 195 (50.23 %), and Estht 198 (36.25 %). This demonstrated a clear variation in callus formation potential of the three arugula varieties.

Var. Estht 201 demonstrated the maximum shoot induction percentage of 67.45 %, followed by Var. Estht 195 displaying 55.37 % (Figure 1), and Var. Estht 198 indicated 53.0

% shoot induction. This highlights variations in shoot proliferation potential across different arugula varieties (Table 1).

Var. Estht 201 produced the highest number of leaves with an average of 19.73, followed by Var. Estht 195 with average of 12.25 leaves per explant, and Var. Estht 198 had 9.75 leaves per explant.

Var. Estht 201 displayed the tallest plants with an average height of 7.50 cm, followed by Var. Estht 198 at 5.25 cm, and Var. Estht 195 at 5.00 cm. This indicated significant variability among arugula varieties in terms of vertical growth.

Table 1. Differences among varieties for regeneration parameters using 0.1 mg L^{-1} NAA and 0.25 mg L^{-1} BAP added to MS medium after 4 weeks of culture

Varieties	**Callus induction % after 3 weeks of culture** **	**Shoot induction %** **	**Number of leaves per explant** *	**Plant height (cm)** *
Estht 201	61.83 a	67.45 a	19.73 a	7.50 a
Estht 195	50.23b	55.37 b	12.25 b	5.00 b
Estht 198	36.25c	53.00 c	9.75 c	5.25 b

**All values shown by different small letters in a single column are statistically different using LSD test at 0.01 level of significance
*All values shown by different small letters in a single column are statistically different using LSD test at 0.05 level of significance

3.2. Effect of blends of 0.1 mg L^{-1} NAA-0.25 mg L^{-1} BAP on regeneration from variety Estht 195, Estht 198, and Estht 201 after 6 weeks of culture

The treatment means showed significant differences for callus induction, shoot regeneration percentage and plant height at $p<0.01$ level. The differences between the average values of the number of leaves per explant were not found to be significant. The second count of callus induction and shoot regeneration was noted after 6 weeks of initial culture. Var. Estht 201 exhibited the highest percentage of callus induction (74.05 %, Figure 2), followed by Var. Estht 195 (50.16 %), and Var. Estht 198 (45.33 %). This consistent parallel trend after 6 weeks of culture suggested that Var. Estht 201 maintained a superior ability to form callus tissues compared to the other varieties (Table 2).

Var. Estht 201 also exhibited the highest shoot induction percentage of 69.9 % (Figure 1), followed by Var. Estht 195 with an induction percentage of 62.8 %, and Var. Estht 198 with shoot induction percentage of 57.0 %. Once again, Var. Estht 201 demonstrated a consistent trend of vigorous shoot induction compared to the other two varieties.

Var. Estht 201 displayed the tallest plants of 9.6 cm, followed by Var. Estht 195 (6.0 cm), and Var. Estht 198 (5.88 cm). This suggested that Var. Estht 201 maintained superior vertical growth compared to the other varieties used in the study.

Table 2. Differences among varieties for regeneration parameters using 0.1 mg L^{-1} NAA and 0.25 mg L^{-1} BAP on MS medium after 6 weeks of culture

Variety	Callus induction % **	Shoots induction %**	Number of leaves per explant ns	Plant Height (cm) **
Estht 201	74.05 a	69.90 a	13.00	9.60 a
Estht 195	50.16 b	62.80 b	11.00	6.00 b
Estht 198	45.33 c	57.00 c	10.25	5.88 c

**All values shown by different small letters in a single column are statistically different using LSD test at 0.01 level of significance
ns non significant

The findings of the Week 6 analysis reconfirm the different significant effect of the same hormonal treatment on different types of arugula. The continued superior performance of Var. Estht 201 in callus formation, leaf production, plant height, and shoot development underscores its potential as a preferred variety for micropropagation.

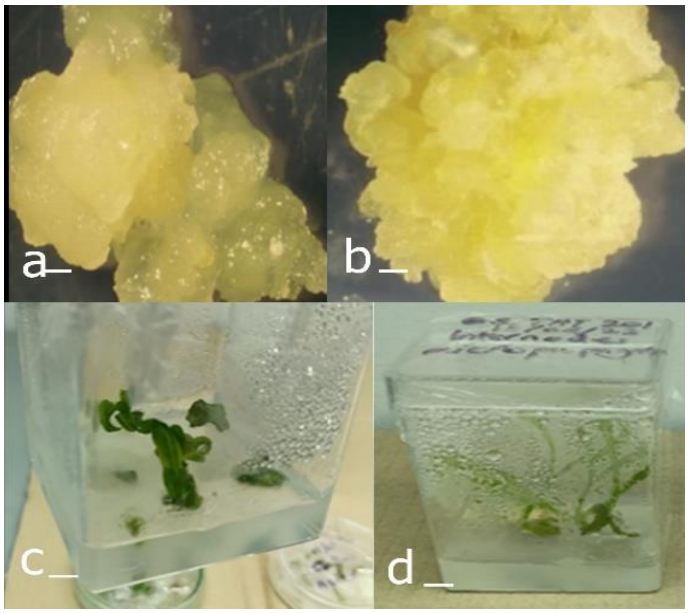

Figure 1. Plant regeneration on different explant of arugula. Callus formation after 1 and 6 weeks of tissue culture in same sequence (bar = 2mm). Indirect plant regeneration after 4 - 6 weeks of tissue culture (bar = 5mm) using Var. Estht 201

This study suggests inherent genetic differences among the varieties, which could be exploited to select superior breeding lines or optimize tissue culture protocols. These finding emphasizes the importance of personalized treatment regimens to maximize micro propagation success across diverse arugula varieties.

Understanding these temporal dynamics is essential for optimizing cultivation protocols and predicting long-term growth outcomes. Moreover, the significant influence of hormonal treatments suggests potential strategies for enhancing arugula cultivation efficiency through targeted hormonal interventions. By understanding the interplay between variety genetics and treatment effects, practitioners can optimize protocols to achieve desired growth outcomes in arugula production. Nagar and Mekawi (2015), have found that there are significant differences between two different arugula genetic types. Callus formation was better in cotyledon explants than in stem explants, induced in MS medium containing different combinations of NAA and BA as well as 2,4-D and Kinetin. Hunaish and Almasoody (2020), used hypocotyl, stems and cotyledon leaves as explants in their research. The highest dry weight they obtained from callus was obtained from cotyledon leaf explants.

The study contributes to the development of more effective micropropagation strategies advancement of arugula breeding and production practices.

4. Conclusion

The study highlights the significant variability in growth performance among 3 arugula varieties, with Var. Estht 201 consistently exhibiting superior characteristics compared to Var. Estht 195 and Var. Estht 198. This underscores the importance of genetic diversity in arugula breeding programs. The impact of hormonal treatments on callus formation and shoot development reaffirms the

crucial role of varieties in modulating physiological processes during arugula micro propagation.

Overall, this analysis provides valuable insights for arugula breeders, growers, and researchers, offering potential strategies for enhancing cultivation efficiency and advancing breeding programs.

Declaration of Author Contributions

The authors declare that they have contributed equally to the article. All authors declare that they have seen/read and approved the final version of the article ready for publication.

Declaration of Conflicts of Interest

All authors declare that there is no conflict of interest related to this article.

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