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Influence of plant growth regulators on *in vitro* germination and micropropagation of *Asparagus officinalis* L.

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ABSTRACT

The germination and micropropagation of *Asparagus officinalis* L. remain understudied, despite its nutritional and medicinal value. This study focused on the influence of plant growth regulators 6-benzylaminopurine (BAP), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) on *in vitro* propagation efficiency. Two approaches were used: seed germination and micropropagation via *in vitro* nodal explants were cultivated on MS medium with varying concentrations of BAP (0.5, 1, or 1.5 mg/L), either individually or combined with 1 mg/L NAA. MS medium supplemented with NAA showed the highest germination rate (76.67%) and optimal growth, producing 3.33 shoots per explant, 8 cm shoot length, 9.66 cm root length, and 9.33 lateral roots. Media supplemented with 2,4-D had a 66.67% germination rate, 3 shoots per explant, 8.33 cm shoot length, and 25 lateral roots. BAP resulted in moderate shoot formation (1.33 shoots per explant) with no lateral root induction. Control explants produced a single shoot. For micropropagation, the control and BAP at 0.5 mg/L resulted in optimal microcutting success rate (55.2%), with 2.06 shoots per explant, 3.34 cm shoot length, and 1 leaves. Higher concentrations of BAP (1.0 and 1.5 mg/L) reduced shoot and leaf development. The combination of 1.5 mg/L BAP with 1 mg/L NAA increased the microcutting success rate to 66.7%, although shoot length (0.86 cm) and leaf count (1.53) were reduced. This study is limited to *in vitro* conditions and a specific range of plant growth regulators concentrations. Further research is needed to explore environmental factors in *ex vitro* culture. These findings provide an efficient protocol for enhancing asparagus propagation, offering practical benefits for horticultural production, conservation, and pharmaceutical applications. This study presents original data on the effect of specific plant growth regulators on both germination and micropropagation, revealing the potential of NAA, 2,4-D and BAP treatments in optimizing *A. officinalis* development under controlled conditions.

Keywords: *Asparagus officinalis* L., plant growth regulators, germination, micropropagation, root development.

INTRODUCTION

In vitro culture is a widely utilized and crucial technique for the efficient and large-scale production of plants, offering notable benefits for propagating species, especially those with valuable genetic traits or difficulties in natural reproduction (Loyola-Vargas and Ochoa-Alejo 2024; Thakur et al., 2024). Among these species, asparagus (*Asparagus officinalis* L.), a horticultural crop that is both nutritious and medicinal, benefits greatly from this approach because of its limited propagation by seeds and the difficulties associated with propagation by cuttings (Regalado et al., 2015; Iqbal et al., 2017). The utilization of *in vitro* culture significantly improves

propagation capabilities, and *in vitro* cultivation of asparagus depends largely on the optimal use of growth regulators, particularly cytokinins and auxins, which are important for the regulation of cell division, shoot elongation, and root initiation (Štajner 2012; Encina and Regalado 2022).

In *in vitro* culture, cytokinins such as 6-benzylaminopurine (BAP) are widely applied to stimulate shoot proliferation in various crops, their effectiveness is highly dependent on their concentration, culture conditions, and interactions with other plant growth regulators, such as auxins (Aremu et al., 2020; Li et al., 2021). Low to moderate concentrations of BAP are generally associated with increased cell division and shoot proliferation, whereas higher concentrations can lead to inhibitory effects, disrupting bud growth and elongation (Madumali et al., 2019; Aworunse et al., 2019). Auxins, including naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), play crucial roles in regulating cell elongation and inducing root formation (Yan et al., 2014). However, a significant gap remains in the understanding of how BAP and NAA interact synergistically or antagonistically to enhance asparagus propagation through micropropagation, which is crucial given the crop's importance in both agriculture and pharmaceuticals. Although some studies have explored the effects of BAP and NAA on other species, research on their combined impact on asparagus officinalis seed germination is scarce, leaving unanswered questions about their interactions.

Despite studies on the effect of growth regulators on various species has been studied in *in vitro* cultures, few studies have investigated the interaction between BAP and NAA in *A. officinalis*. This lack of knowledge on the optimal concentrations and interactions of these plant growth regulators represents a significant gap in the field. The germination of asparagus seeds depends on several factors, such as light, growth medium, and physical state (Abid et al., 2025). However, the exact role of plant growth regulators in optimizing this process remains unclear. This research sought to optimize the effects of plant growth regulators on the *in vitro* germination and micropropagation of *A. officinalis* through node culture. By exploring the mechanistic roles of plant growth regulators, this study seeks to elucidate the regulatory pathways that modulate shoot proliferation and germination efficiency. Furthermore, the research will examine synergistic and antagonistic interactions between specific plant growth regulators concentrations to determine the optimal hormonal balance for enhanced regeneration and propagation. The hypothesis of this research is that synergistic combinations of plant growth regulators will improve the propagation efficiency of *A. officinalis*, leading to more robust plantlets and higher yields. These findings are expected to inform practical and effective cultivation strategies, contributing to sustainable asparagus production.

MATERIALS AND METHODS

Plant material

Seeds of the Argenteuil variety of *Asparagus officinalis* were exposed to a rapid soaking process in 70% (v/v) ethanol for 60 seconds, than 60% (v/v) sodium hypochlorite solution for five minutes. Following this process, the seeds underwent a thorough cleaning process, involving three rinses in sterile distilled water, followed by drying on sterile filter paper. The seeds were then sown in the corresponding culture media and placed in a tissue culture room (Figure 1). Once the seeds had germinated and the seedlings reached a sufficient size, 1 cm stem segments (axillary buds) were excised for use as explants**.** The explants used in this study were derived from asparagus plantlets after *in vitro* germination in media devoid of growth regulators. All operations were conducted under sterile conditions in a laminar flow hood.

Culture medium

Axillary bud explants from *in vitro* plants were transferred to culture tubes containing 10 mL of Murashige and Skoog, 1962 (MS) culture medium. This medium was prepared by adding 30 g/L sucrose and all the elements required for seedling growth, such as macroelements, microelements. The pH of the medium was calibrated to 5.7 ± 0.1 using a sodium hydroxide (NaOH) solution or hydrochloric acid (HCl). Agar (7 g/L) was added to the MS media to solidify the media. The sterilization of the medium was accomplished through autoclaving at 121 °C for 20 minutes. Four culture media were tested in the germination study: a hormone-free medium as a control and three media enriched with 2 mg/L of each growth regulator, 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneacetic acid (NAA). For the micropropagation

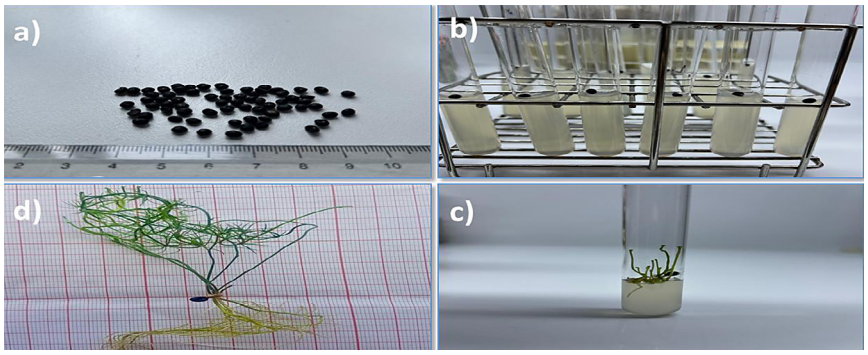

Figure 1. *In vitro* culture of *Asparagus officinalis* L.: (a) seeds before sterilization, (b) seeds placed on culture medium in sterile conditions, (c) germinated seedlings growing *in vitro*, (d) morphological measurements of plantlets after five weeks of *in vitro* culture

medium, seven combinations of growth regulators were utilized; the following concentrations of BAP were used: 0, 0.5, 1, and 1.5 mg/L with or without 1 mg/L NAA.

Growing conditions

All the cultures were incubated in a tissue culture room maintained at 25 ± 2 °C with a photoperiod of 16 h of light and 8 h of darkness under fluorescent lights, which provided an intensity of 45 μmol $m^{-2}s^{-1}$. The explants were inspected regularly to ensure optimal development.

Statistical analysis

Descriptive statistics and confidence intervals

Descriptive statistics, including mean/proportions, standard deviations (SD), standard errors (SE) and confidence intervals (CI) were calculated for each treatment group.

Analysis of binary categorical variables

Specifically, for binary variables such as "germination rate", "microcutting success rate under BAP treatment", and "microcutting success rate under combined BAP and NAA treatment", where the outcome for each observation unit was 0 or 1, the standard deviation and standard error were calculated respectively using the formulas:

$$SD = \sqrt{\hat{p}(1-\hat{p})} \tag{1}$$

$$SE = \sqrt{\frac{\hat{p}(1-\hat{p})}{n}} \tag{2}$$

where: $\hat{p}$ – sample proportion, n – total number of observations.

It should be noted that the 95% Wilson CIs (Daniel, 2013) were calculated for "germination rate", "microcutting success rate under BAP treatment", and "microcutting success rate under combined BAP and NAA treatment". This method was chosen due to its robustness for estimating proportions, especially with small sample sizes or when proportions are close to 0 or 1. Wilson Confidence Intervals (Daniel, 2013) were calculated using the following formula:

$$CI = \frac{\hat{p} + \frac{z^2}{2n} \pm z \cdot \sqrt{\frac{\hat{p}(1-\hat{p})}{n} + \frac{z^2}{4n^2}}}{1 + \frac{z^2}{n}} \tag{3}$$

where: z – critical value of the standard normal distribution (for 95% CI, z = 1.96), $\hat{p}$ – sample proportion, n – total number of observations.

Logistic regression analysis

The logistic regression (Ranganathan, 2017) is ideally suited for modeling the probability of binary events, as it estimates the likelihood of belonging to one of two categories. Using logistic regression, categorical independent variables can be evaluated, allowing for the quantitative determination of the influence of different plant growth regulator concentrations (categorical variables) on the probability of success, by utilizing dummy variables with a control group as the reference.

The output provides interpretable Odds Ratios (ORs), which quantify how many times higher or lower the odds of success are in one group compared to the reference. For these odds ratios, 95% confidence intervals are calculated, providing a range within which the true odds ratio is

likely to fall. This allows for an assessment of the precision of the effect estimate and its statistical significance (if the interval does not cross 1). The overall model significance was assessed using the Likelihood Ratio Test. For pairwise comparisons between groups, adjustments for multiple testing (Holm's method) were applied to control the risk of Type I errors. For calculating the logistic regression analysis, the *statsmodels* library in Python was utilized, specifically its formula.api module for model specification. Data preprocessing and organization were performed using the *pandas* library. Calculations of odds ratios and other mathematical operations were carried out with *numpy*. Hypothesis testing, including individual comparisons and pairwise group comparisons, was conducted using the *Wald test* (with a χ^2 statistic) within the *statsmodels* library. For p-value correction in multiple comparisons, methods such as *Holm* ('holm') were applied via the *multipletests* function from the *statsmodels.stats.multitest* module.

Analysis of absolute metric variables

For absolute metric variables, such as microshoot length and leaf counts, we encountered two fundamental challenges that precluded the use of standard parametric tests (e.g., t-intervals, ANOVA). First, non-normal distributions: biological growth metrics frequently exhibit right-skewed patterns, violating the normality assumption required for many parametric methods.

Second, heteroscedasticity: the variance of the data often scaled with mean values ($\sigma \propto \mu$), indicating non-homogeneous variances across groups. To address these issues while maintaining the interpretability of measurements in their original absolute units, we implemented a Percentile Bootstrap method (Efron, 1981) with 10.000 resamples. This method provided distribution-free interval estimates that preserved the original measurement units (e.g., centimeters for lengths, counts for leaves). It effectively accommodated heterogeneous variance structures and remained robust against outliers and data skewness. This approach was applied when bootstrap diagnostics indicated persistent asymmetry or other issues that might affect the robustness of the bootstrap intervals. For the determination of the 95% confidence intervals, we used the Python programming environment (version 3.10), utilizing the NumPy library (for numerical operations), SciPy (for statistical functions), and scikit-learn (specifically sklearn.utils.resample for resampling procedures).

Non-parametric comparisons (Kruskal-Wallis H-test)

To assess statistically significant differences between independent groups when data did not meet the assumptions of parametric tests, we also employed the non-parametric Kruskal-Wallis H-test. This test is a non-parametric analogue to one-way ANOVA, allowing for the detection of differences in distributions among groups without assuming data normality or homogeneity of variances. The Kruskal-Wallis H-test is more powerful than the Median test, less sensitive to outliers than parametric methods, and suitable for analyzing ordinal scale data. The analysis procedure involved ranking all observations from the smallest to the largest value and computing the H statistic using the formula:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(N+1) \qquad (4)$$

where: N – total number of observations, n_i – number of observations in the i-th group, R_i – sum of ranks in the i-th group, k – number of groups.

A significant result ($p < 0.05$) indicates differences between at least two groups.

Software

Statistical calculations and analyses were performed using the Python programming environment (version 3.10), IBM SPSS Statistics 21 software and Microsoft Excel 2010.

RESULTS

Germination medium

Effects of plant growth regulators on germination

These findings demonstrated that different plant growth regulators treatments had varying effects on the rate of seed germination, as shown in Figure 2, Figure 3 and Table 1. The control had a germination rate of 36.7% ± 0.08 percentage points, indicating a low level of germination. BAP treatment did not improve germination at a rate of 53.3% ± 0.09. In contrast, treatment with NAA

had a significant effect on germination (76.67% ± 0.07), making this medium the most effective treatment. The 2,4-D treatment improve germination rate of 66.67% ± 0.08, indicating an improvement over the control and BAP treatments.

Odds ratios were calculated relative to the control group for treatments (BAP, 2,4-D, NAA). The overall logistic regression model evaluating the effect of plant growth regulators on germination success was statistically significant (Likelihood ratio test: χ^2 (3) = 11.33, p = 0.010). Post-hoc comparisons, adjusted for multiple testing using Holm's method, revealed significant differences among the treatment groups Specifically, treatment with NAA significantly improved the germination rate compared to the control group, showing an Odds ratio of 5.675 (95% CI: 1.841–17.494), which was statistically significant (p = 0.015). In contrast, BAP treatment did not lead to a statistically significant improvement in germination rate when compared to the control group (Odds ratio = 1.974, 95% CI: 0.703–5.543, Holm p = 0.590). Similarly, while 2,4-D treatment showed a tendency for increased germination, its effect was not statistically significant after multiple comparison adjustment (Odds ratio = 3.455, 95% CI: 1.195–9.990, Holm p = 0.111). No statistically significant differences were observed among the BAP, 2,4-D, and NAA treatment groups in terms of germination success after multiple comparison adjustments.

Effects of plant growth regulators on seedling size and the root number and length

The development of the aerial and root portions of the seedlings depended on the use of PRGs (Kruskal-Wallis H-test, H = 6.89; p = 0.075 and H = 7.50; p = 0.057, respectively) (Figure 4 and Figure 5). Maximum stem lengths of 8 ± 0 cm and 8.33 ± 3.51 cm were obtained with NAA and 2,4-D, respectively. For the roots,

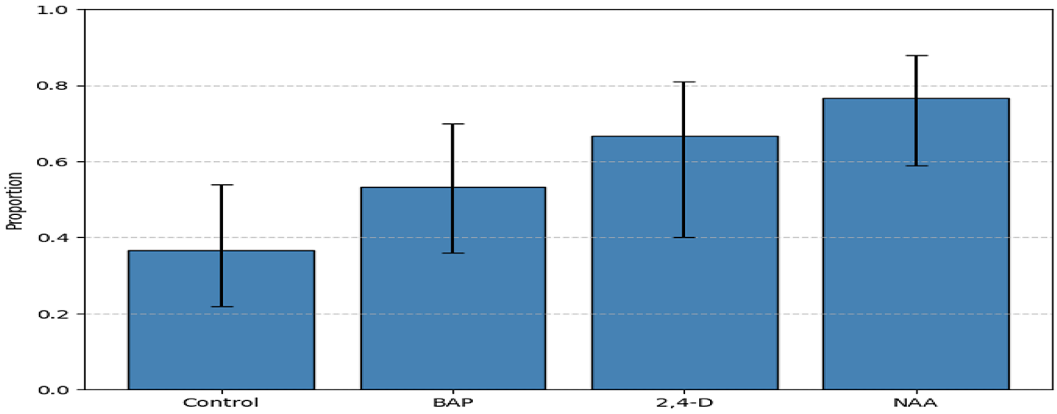

Figure 2. Effects of growth regulators on the germination of *Asparagus officinalis* L. seeds with 95% confidence intervals

Figure 3. Seedlings of *Asparagus officinalis* L. germinated after 5 weeks of cultivation

Table 1. Descriptive statistics and confidence intervals (binary categorical variables) for germination and microcutting success rate under treatments with 1-Naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BAP), and 2,4-Dichlorophenoxyacetic acid (2,4-D)

Parameter	Treatment, mg/L	Proportion $\hat{p}$	SD	SE	95% Wilson CI	Overlap (relative to control CI), %
Germination rate	NAA	0.767	0.423	0.077	[0.59, 0.88]	0
	BAP	0.533	0.499	0.091	[0.36, 0.70]	56.25
	2,4-D	0.667	0.471	0.086	[0.40, 0.81]	43.75
	Control	0.367	0.482	0.088	[0.22, 0.54]	
	Total	0.583	0.493	0.045	[0.49, 0.67]	
Microcutting success rate under BAP treatment	0 mg BAP	0.552	0.497	0.092	[0.38; 0.72]	
	0,5 mg BAP	0.552	0.497	0.092	[0.38; 0.72]	100
	1 mg BAP	0.448	0.497	0.092	[0.28, 0.62]	70.59
	1,5 mg BAP	0.241	0.428	0.079	[0.12, 0.42]	11.76
	Total	0.448	0.497	0.046	[0.36, 0.54]	
Microcutting success rate under combined BAP and NAA treatment	0,5 mg BAP+ 1 mg NAA	0.400	0.490	0.126	[0.2; 0.64]	
	1 mg BAP+ 1 mg NAA	0.200	0.400	0.103	[0.07, 0.45]	
	1,5 mg BAP+ 1 mg NAA	0.667	0.442	0.114	[0.48, 0.89]	
	Total	0.444	0.074	0.074	[0.31, 0.59]	

the NAA medium had the best results, with the roots reaching 9.66 ± 10.69 cm, followed by the 2,4-D (8.66 ± 1.15 cm) and hormone-free media (4 ± 2 cm). The medium supplemented with BAP resulted in the shortest root length, at 1.66 ± 0.57 cm.

Statistical analysis of the number of stems (Kruskal-Wallis H-test, H = 8.35; p = 0.039) revealed that the number of stems varied depending on the regulator. The NAA medium resulted in the greatest number of stems, with an average of 3.33 ± 0.88 stems per explant, followed by 2,4-D medium (3 ± 0.57 stems) and BAP (1.33 ± 0.33 stems) (Figure 6). The control medium had an average of one stem ± 0. For lateral roots (Kruskal-Wallis H-test, H = 6.33; p = 0.096), media containing 2,4-D favored the formation of 25 ± 12.58 lateral roots, followed by media supplemented with NAA (9.33 ± 1.20 roots) and hormone-free media (3.66 ± 1.20 roots). No lateral roots developed in media supplemented with BAP (Table 2).

Micropropagation

Effect of BAP alone

Figure 7, Figure 8 and Table 1 shows the effects of different plant growth regulator treatments on the success rate of microshoot and the

Figure 4. Effects of growth regulators on seedling development: (a) 2,4-dichlorophenoxyacetic acid (2,4-D), (b) BAP, (c) control and (d) naphthaleneacetic acid (NAA)

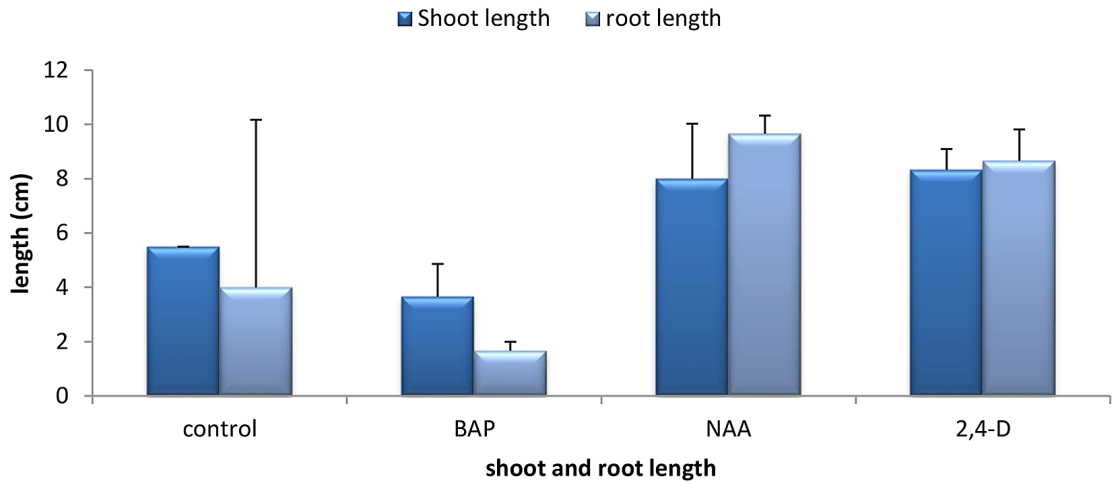

Figure 5. Effects of growth regulators on the size and root development of *Asparagus officinalis* L. seedlings after 5 weeks of germination

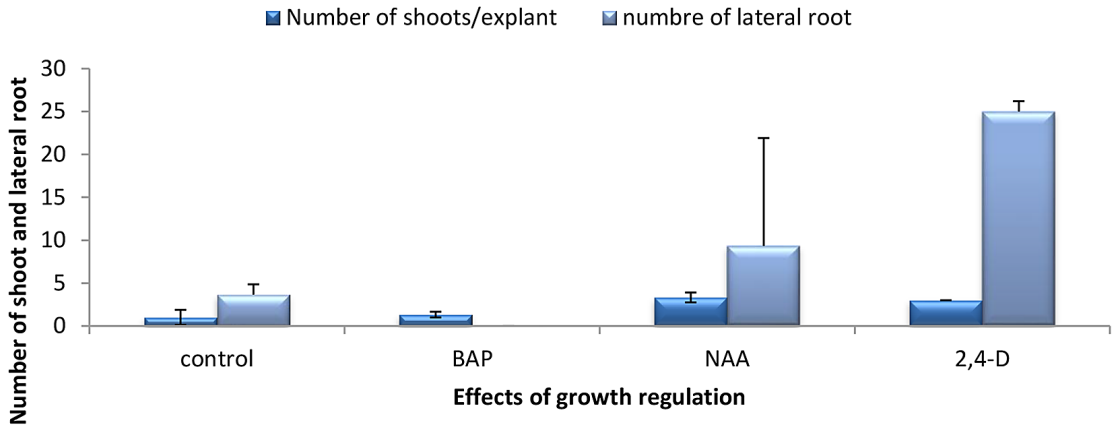

Figure 6. Effects of growth regulation on the number of stems and lateral roots of *Asparagus officinalis* seedlings after 5 weeks of germination

development of asparagus shoots in *in vitro* culture. The data revealed that shoot proliferation was maximal (55.2% ± 0.09%) at a BAP concentration of 0 and 0.5 mg/L. However, a progressive decrease was observed with increasing concentration, reaching a significant minimum of 24.1 ± 0.07% at 1.5 mg/L.

Odds ratios were calculated relative to the control group for the treatments (0.5 mg/L BAP, 1 mg/L BAP, 1.5 mg/L BAP). The overall logistic regression model evaluating the effect of plant growth regulators on microcutting success rate under BAP treatment was statistically significant (Likelihood ratio test: χ^2 (3) = 7.84, p = 0.0495). Post-hoc comparisons, adjusted for multiple testing using Holm's method, did not reveal statistically significant individual differences among the treatment groups. Specifically, compared to the control group shown: Treatment with 0.5 mg/L BAP did not lead to a statistically significant change in microcutting success rate (Odds ratio = 1.000, 95% CI: 0.355–2.815, Holm p = 1.000). Treatment with 1 mg/L BAP also showed no statistically significant effect on microcutting success rate (Odds ratio = 0.660, 95% CI: 0.235–1.858, Holm p = 1.000). Treatment with 1.5 mg/L BAP showed a tendency towards a decrease in the odds of microcutting success rate (Odds ratio = 0.258, 95% CI: 0.084–0.794), but this effect was not statistically significant after multiple comparison adjustment (Holm p = 0.1088). Furthermore, no statistically significant differences were observed among the BAP treatment groups themselves (0.5 mg BAP, 1 mg/L BAP, and 1.5 mg/L BAP) regarding microcutting success rate after multiple comparison adjustments: 0.5 mg/L BAP versus 1 mg BAP (Odds ratio = 1.515, Holm p = 1.000); 0.5 mg/L BAP versus 1.5 mg/L BAP (Odds ratio = 3.868, Holm p = 0.1088); 1 mg/L BAP versus 1.5 mg/L BAP (Odds ratio = 2.554, Holm p = 0.4060).

Table 2. Descriptive statistics and confidence intervals (Absolute Metric Variables) for the effects of NAA, BAP, and 2,4-Dichlorophenoxyacetic acid (2,4-D) on morphological parameters during germination and micropropagation stages

Parameter	Treatment, mg/L	Mean	SD	SE	Min	Max	95%CI
Number of stems formed during germination	NAA	3.333	1.527	0.882	2.0	5.0	[2.00; 5.00]
	BAP	1.333	0.577	0.333	1.0	2.0	[1.00, 2]
	2,4-D	3.000	1.000	0.577	2.0	4.0	[2.00; 4]
	control	1.000	0.000	0.000	1.0	1.0	[1.00; 1.00]
	Total	2.167	1.331	0.386	1	5	[1.50; 2.92]
Shoot length at the germination stage	NAA	8.000	0.000	0.000	8.0	8.0	[8.00; 8.00]
	BAP	3.667	2.081	1.201	2.0	6.0	[2.0; 6.0]
	2,4-D	8.333	3.512	2.028	5.0	12.0	[5.0; 12.0]
	control	5.500	1.323	0.764	4.5	7.0	[4.5; 7.0]
	Total	6.375	2.706	0.781	2.0	12.0	[4.96; 7.88]
Root length at the germination stage	NAA	9.667	10.693	6.173	3	22	[3.0; 22.0]
	BAP	1.667	0.577	0.333	1	2	[1.0; 2.0]
	2,4-D	8.667	1.155	0.667	8	10	[8.0; 10.0]
	control	4.000	2.000	1.155	2	6	[2.0; 6.0]
	Total	6.000	5.799	1.674	1	22	[3.33; 9.50]
Number of lateral roots formed during germination	NAA	9.333	2.082	1.202	7	11	[7.00; 11.00]
	BAP	0.000	0.00	0.00	0	0	[0.00; 0.00]
	2,4-D	25.00	21.794	12.583	0	40	[0.00; 40.00]
	control	3.667	2.082	1.202	2	6	[2.00; 6.00]
	Total	9.500	13.688	3.951	0	40	[3.00; 17.67]
Stem length of microshoots under BAP treatment	0 mg BAP	1.629	2.414	0.448	0.0	10.5	[0.85; 2.59]
	0,5 mg BAP	3.345	5.752	1.068	0.0	20.0	[1.48; 5.59]
	1 mg BAP	0.966	2.256	0.419	0.0	8.0	[0.26; 1.86]
	1,5 mg BAP	0.717	2.162	0.401	0.0	9.5	[0.09; 1.59]
	Total	1.41	2.81	0.26	0.0	20.0	[1.05; 2.36]
Number of leaves per microshoot under BAP treatment	0 mg BAP	1.517	3.398	0.631	0	14	[0.45; 2.9
	0,5 mg BAP	1.000	2.330	0.433	0	11	[0.31; 1.93]
	1 mg BAP	1.552	5.883	1.093	0	31	[0.1; 4.0]
	1,5 mg BAP	1.172	2.904	0.539	0	11	[0.28; 2.31]
	Total	1.31	3.83	0.356	0	31	[0.7; 2.09]
Number of shoots per explant derived from microshoots under BAP treatment	0 mg BAP	0.724	1.533	0.285	0	8	[0.31; 1.34]
	0,5 mg BAP	2.069	4.456	0.827	0	20	[0.76; 3.86]
	1 mg BAP	0.483	0.574	0.107	0	2	[0.28; 0.69]
	1,5 mg BAP	0.414	1.211	0.225	0	6	[0.07; 0.9]
	Total	0.922	2.51	0.233	0	20	[0.53, 1.42]
Number of shoots per explant under combined BAP and NAA treatment	0,5 mg BAP+ 1 mg NAA	14.400	18.749	4.841	0	48	[5.87; 23.73]
	1 mg BAP+ 1 mg NAA	0.333	0.724	0.187	0	2	[0.0; 0.73]
	1,5 mg BAP+ 1 mg NAA	0.800	0.676	0.175	0	2	[0.47; 1.13]
	Total	5.178	12.478	1.86	0	48	[1.89; 9.0]
Leaf formation under combined BAP and NAA treatment	0,5 mg BAP+ 1 mg NAA	1.333	2.820	0.728	0	10	[0.20; 2.87]
	1 mg BAP+ 1 mg NAA	0.467	1.356	0.350	0	5	[0.00, 1.27]
	1,5 mg BAP+ 1 mg NAA	1.533	2.356	0.608	0	7	[0.53, 2.80]
	Total	1.111	2.259	0.337	0	10	[0.51; 1.80]

Stem length under combined BAP and NAA treatment	0,5 mg BAP+ 1 mg NAA	3.600	4.687	1.210	0.0	12.0	[1.47; 5.93]
	1 mg BAP+ 1 mg NAA	1.000	2.204	0.569	0.0	7.0	[0.0; 2.2]
	1,5 mg BAP+ 1 mg NAA	0.867	1.950	0.503	0.0	6.5	[0.07; 1.93]
	Total	1.822	3.371	0.503	0.0	12.0	[0.91; 2.84]

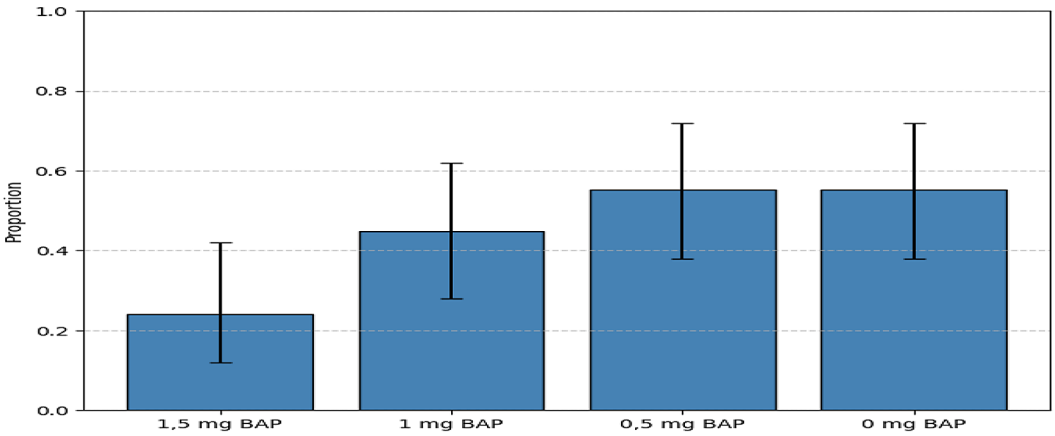

Figure 7. Effects of different concentrations of BAP on the success rate of micropropagation in *Asparagus officinalis* L. with 95% CI

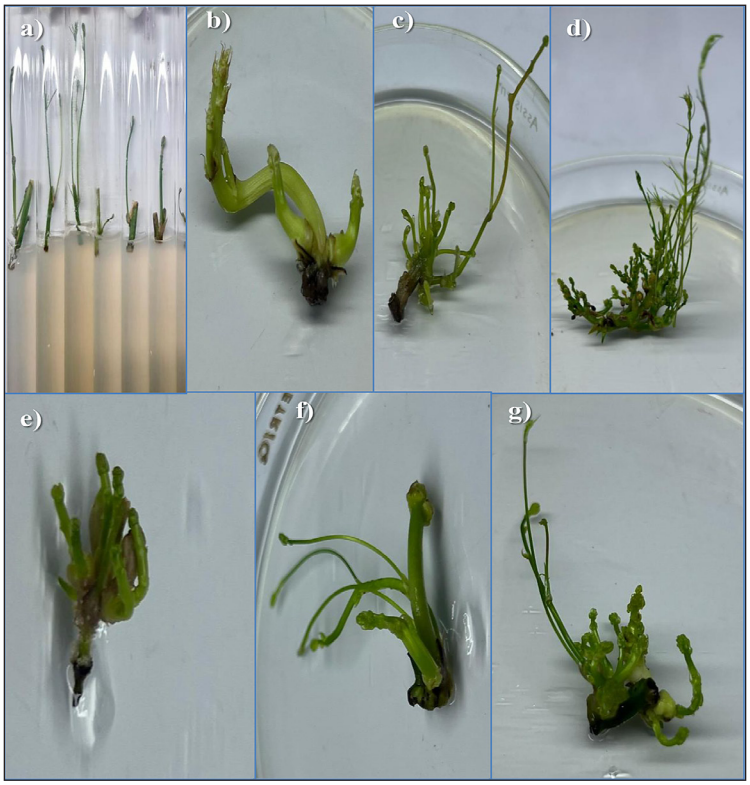

Figure 8. Micropropagation of *Asparagus officinalis* L. *in vitro* plants in different culture media: (a) media enriched with 1.5 mg/L 6-benzylaminopurine BAP, (b) media supplemented with 1 mg/L 6-benzylaminopurine BAP and 1 mg/L naphthaleneacetic acid NAA, (c) control media without growth regulators, (d) media supplemented with 0.5 mg/L 6-benzylaminopurine BAP, (e) media supplemented with 1.5 mg/L 6-benzylaminopurine BAP and 1 mg/L naphthaleneacetic acid NAA, (f) media supplemented with 1 mg/L 6-benzylaminopurine BAP, and (g) media supplemented with 0.5 mg/L 6-benzylaminopurine BAP and 1 mg/L naphthaleneacetic acid NAA

The number of shoots per explant also varied according to the BAP dose (Kruskal-Wallis H-test, H = 5.17; p = 0.159). The 0.5 mg/L concentration was the most efficient, producing 2.06 ± 0.82 shoots per explant, which was greater than the 0, 1, and 1.5 mg/L concentrations, which produced only 0.72 ± 0.28, 0.48 ± 0.10, and 0.41 ± 0.22 shoots per explant, respectively. However, these results must be interpreted in light of the shoot length (Kruskal-Wallis H-test, H = 12.83; p = 0.005) (Figure 9). Indeed, the 0.5 mg/L concentration favored the highest shoot length (3.34 ± 1.06 cm), whereas higher concentrations, such as 1 and 1.5 mg/L, led to a significant reduction in length (0.96 ± 0.41 and 0.71 ± 0.40), respectively.

The number of leaves showed a pattern similar to shoot length (Kruskal-Wallis H-test, H = 0.611; p = 0.89). In the control group (0 mg/L of BAP), the mean number of leaves was 1.51 ± 0.63 (Figure 10). This decreased to 1 ± 0.43 at 0.5 mg/L BAP. The highest leaf number was observed at 1 mg/L (1.52 ± 1.09), followed closely by 1.5 mg/L (1.17 ± 0.53).

Effect of the interaction between BAP and NAA

The data revealed distinct effects depending on the concentration of growth regulators. Statistical analysis revealed that the observed differences in microshoot success rates were statistically significant (Figure 11). These results indicate that there were no statistically significant differences among the groups tested for shoot length (H = 3.09; p = 0.21), number of leaves (H = 2.57; p = 0.27), or number of shoots per explant (H = 4.35; p = 0.11) under the combined BAP and NAA treatment.

The addition of 0.5 mg/L BAP and 1 mg/L NAA, the microshoot success rate reached 40 ± 0.12% (Table 1), while the number of shoots per explant reached (14.4 ± 4.84 shoots per explant). This combination resulted in the longest shoots (3.06 ±1.21 cm). Furthermore, the statistical results revealed that this combination produced the best results in terms of shoot development, suggesting that this combination is optimal for balanced development.

Odds ratios were calculated relative to the (0.5 mg/L BAP + 1 mg/L NAA) group for the treatments (1 mg/L BAP +1 mg/L NAA; 1.5 mg/L

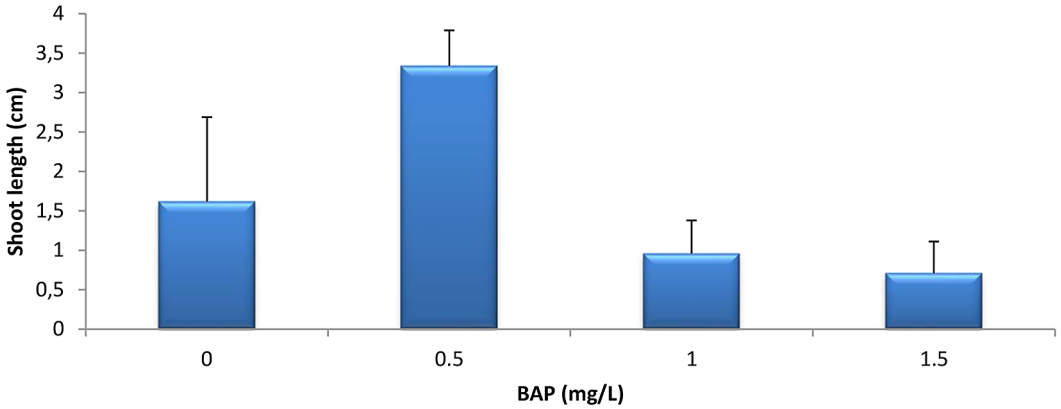

Figure 9. Effects of BAP alone on the stem length of microshoots of A*sparagus officinalis* L.

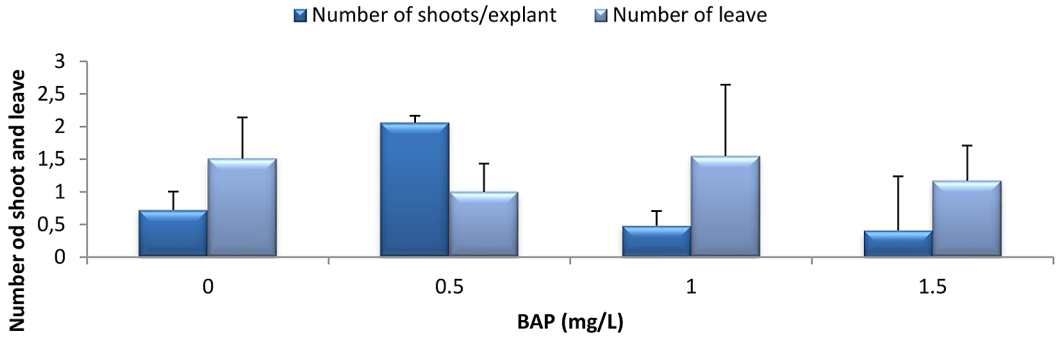

Figure 10. Effects of different concentrations of BAP on the number of shoots per explant and the number of leaves of *Asparagus officinalis* L.

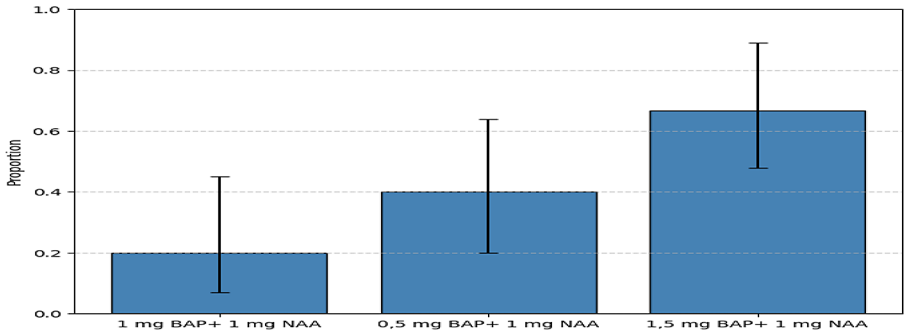

Figure 11. Effects of the interaction between BAP and NAA on the microshoot succes rate of *Asparagus officinalis* L. with 95% CI

BAP +1 mg/L NAA; 1.5 mg/L BAP). The overall logistic regression model evaluating the effect of microcutting success rate under combined BAP and NAA treatment was statistically significant (Likelihood ratio test: χ^2 (3) = 9.23, p = 0.0099). Post-hoc comparisons, adjusted for multiple testing using Holm's method, did reveal statistically significant individual differences among the treatment groups. A direct comparison between the 1.5 mg/L and 1.0 mg/L BAP treatments revealed a statistically significant difference (OR = 0.091; Wald χ^2 = 7.589; p = 0.018; Holm-adjusted p = 0.018), confirming that microshoot success rate was significantly higher at the 1.5 mg/L BAP concentration when combined with 1.0 mg/L NAA. Specifically, the 1.5 mg/L BAP + 1.0 mg/L NAA treatment significantly increased microcutting success rate compared to the 1.0 mg/L BAP + 1.0 mg/L NAA treatment, indicating that a higher BAP concentration enhances microcutting success rate when combined with NAA.

However, when the concentration of BAP was increased to 1 mg/L and NAA was maintained at 1 mg/L, microcutting success rate decreased to 20 ± 0.10%, and the number of shoots per explant decreased considerably (0.33 ± 0.18) (Figure 12). This decrease was accompanied by a reduction in shoot length to 1 ± 0.56 cm (Figure 13).

At a concentration of 1.5 mg/L BAP with 1 mg/L NAA, micrucuting success rate reached highest level of 66.7 ± 0.11%, but the number of shoots per explant remained low at 0.8 ± 0.17. The shoot length significantly decreased to 0.86 ± 0.50 cm, whereas the number of leaves reached a maximum of 1.53 ± 0.60. This combination shows that although proliferation is increased, shoot quality is compromised.

DISCUSSION

Our study examined the effects of the growth regulators BAP, NAA, and 2,4-dichlorophenoxyacetic acid (2,4-D) on the germination of asparagus seedlings. The results of this study revealed significant differences in seed germination rates depending on plant growth regulator treatment. The control group presented the lowest seed germination capacity under standard conditions without plant growth regulator treatment. These observations are in agreement with the observations of Abid et al. (2025), who reported that

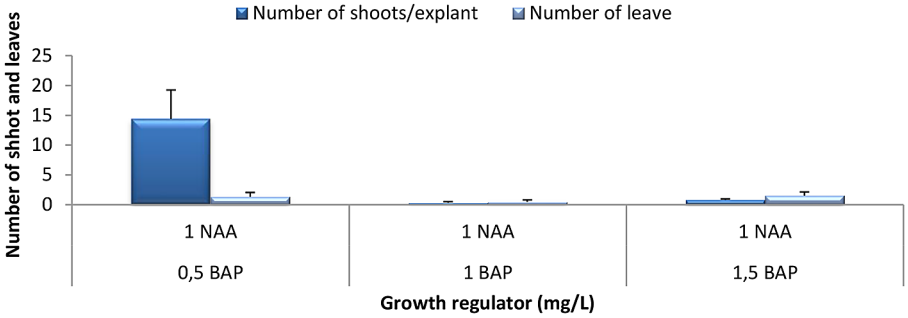

Figure 12. Effects of the interaction effect between BAP and NAA on the number of shoots per explant and the leaf formation in *Asparagus officinalis* L.

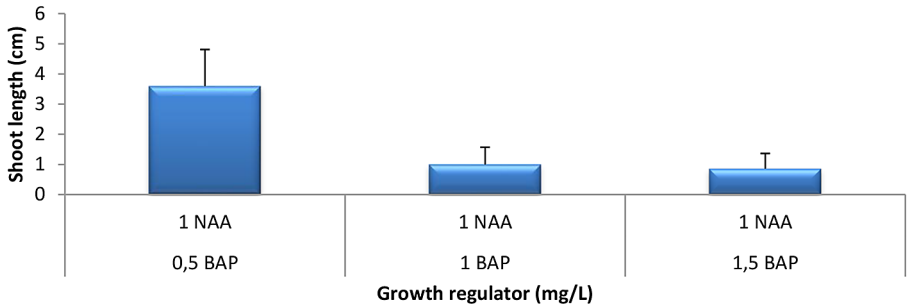

Figure 13. Effects of the interaction between BAP and NAA on stem length in *Asparagus officinalis* L.

untreated asparagus seeds grown in MS media under a photoperiod of 16 h of light presented limited germination rates.

|Our results showed that BAP did not significantly improve germination, this contrasts with Agha et al., (2022), who found BAP most effective compared with the other plant growth regulators tested (2,4-D and NAA) in fenugreek (*Trigonella foenum-graecum*). Such variation may reflect species-specific responses to cytokinins. However, NAA treatment significantly improved germination, which is consistent with studies that have established auxins as key regulators of germination. Our findings are consistent with those of Xing et al. (2023), who reported that NAA promotes germination in soybeans. The 2,4-D treatments also significantly improved the germination rate, this result aligns with the research of Kanmegne and Omokolo (2008), who found that 2,4-D efficacy was slightly lower than that of NAA in *Garcinia kola*. The improvement in seedling growth by NAA and 2,4-D after germination was consistent with the findings of Gallavott (2013), who reported that auxin application promotes stem elongation. Indeed, for *in vitro* cultured plants derived from the germination of media enriched with 2 mg/L BAP, the stem length and number of stems per explant were limited. The variation in germination and growth responses among plants can be attributed to differences in endogenous plant growth regulators. These internally synthesized hormones govern key physiological processes such as cell division, organ initiation, dormancy, germination and ripening.

In vitro micropropagation of asparagus responded best to 0.5 mg/L BAP, which maximally stimulated shoot proliferation and growth. This result is consistent with the work of Monga et al. (2014), who reported that moderate concentrations of BAP promote shoot multiplication in *Ocimum gratissimum*. The crucial role of cytokinins, particularly BAP, in cell division (meresis) and shoot formation has been widely documented in *in vitro* cultures, as confirmed by Arli and Noli (2024) and Ibrahim et al., (2024). At relatively high BAP concentrations, there was a significant decrease in stem regeneration. This phenomenon was also reported by Idei and Kondo (1998), who reported that high BAP levels (2 mg/L) can lead to a decrease in stem proliferation in *Utricularia praelonga*. However, Ilmiyah et al. (2022) reported that high concentrations of BAP do not significantly affect the proliferation of banana plants. These findings suggest that excessive concentrations of BAP may induce physiological stress or disrupt the hormonal balance required for optimal shoot growth, depending on the plant material. Ramayana et al. (2022) reported that high concentrations of cytokinins or auxins can indeed inhibit growth through a hormonal imbalance that slows shoot growth. In terms of the effect of BAP alone on microshoots, treatments with low levels of BAP (0.5 mg/L) yielded the best results in terms of shoot length and number of leaves. Amelia et al. (2020) reported a significant effect on the shoot length and number of leaves of *Melaleuca alternifolia* seedlings at moderate concentrations, and Arafah et al. (2021) reported that BAP had a significant effect on the height of the seedling and the number of leaves in potatoes. In contrast, treatments with higher concentrations of BAP not only reduced proliferation but also led to shorter shoots and a lower number of leaves, indicating an inhibitory effect on overall plant growth. The interaction between BAP and NAA revealed that adding NAA had less pronounced effects than adding BAP alone but still led to a slight growth improvement in certain treatments, particularly when 0.5 mg/L BAP was supplemented with 1 mg/L NAA. NAA is known to play a role in cell elongation

and organogenesis. Jones et al. (2004) and Oh et al. (2014), indicated that the combination of BAP and NAA could have a synergistic effect, notably enhancing auxesis and meresis, which favors better cell organization and differentiation. Pereira et al. (2017) reported that the balance between auxins and cytokinins is essential for optimal growth and that high concentrations of one of these plant growth regulators can have negative effects on cell proliferation, cause phytotoxicity, and inhibit growth.

CONCLUSIONS

This study successfully optimized protocols for both seed germination and micropropagation in *Asparagus officinalis* L., with a focus on hormonal regulation. Auxin treatments with NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) significantly improved germination rates, and resulted in the greatest shoot and root elongation, indicating enhanced early stage vigor. In micropropagation, shoot response was strongly influenced by the concentration of BAP. A concentration of 0.5 mg/L yielded the longest shoots, while a higher concentration (1.5 mg/L) negatively affected microcutting success rates. Notably, when 6-benzylaminopurine was combined with naphthaleneacetic acid, the success rate improved at higher cytokinin levels, although no statistically significant variation was observed in shoot number or shoot length across these treatments. While several regeneration protocols exist for *Asparagus officinalis*, few studies have systematically evaluated the concentration-dependent effects of different plant growth regulators on both seed germination and micropropagation within a unified experimental framework. This study addresses this gap by providing a comprehensive analysis of the individual and combined effects of benzylaminopurine, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid on *in vitro* culture responses. These findings refine existing propagation protocols and offer valuable insights for large-scale propagation and germplasm conservation. Future research should focus on elucidating the molecular mechanisms underlying these hormone interactions to further enhance regeneration efficiency and to facilitate the application of these protocols to other economically important species.

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