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Effect of Moringa Leaf Extract and Zeatin on in Vitro Shoot Induction of Olive Cultivar ‘Gemlik’

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Abstract

The micropropagation of olive (*Olea europaea* L.) is still difficult due to the slow regeneration rate of many cultivars and the high cost of cytokinins employed in tissue culture. The present study aimed to assess the effect of Moringa (*Moringa oleifera* Lam.) leaf extract (MLE) and zeatin on in vitro shoot induction and plantlet development of olive cv. Gemlik. Nodal explants were cultured in a two-factor factorial experiment with four levels of zeatin (0, 1, 2, 3 mg L^{-1}) and three levels of MLE (10, 20, 30 mL L^{-1}) in a completely randomized design with three replications. Days to shoot induction, shoot induction percentage, number of shoots per explant, shoot length, number of leaves, fresh weight, dry weight and chlorophyll content were recorded. The interaction of zeatin and MLE significantly influenced shoot induction percentage, shoot length and plant biomass, whereas both zeatin and MLE significantly affected the measured growth responses ($P \leq 0.01$). The best regeneration efficiency was achieved by 3 mg L^{-1} zeatin and 30 mL L^{-1} MLE, which resulted in the minimum time to shoot induction, maximum shoot induction percentage, maximum shoot proliferation, maximum shoot elongation and maximum plant biomass. It is concluded that 3 mg L^{-1} zeatin combined with 30 ml L^{-1} MLE represents the most effective treatment for enhancing shoot induction, shoot proliferation, shoot elongation, biomass accumulation, and chlorophyll content with no somacolonal variation in olive cv. Gemlik, however 2 mg L^{-1} zeatin with 30 ml L^{-1} MLE being statistically similar and closely comparable, also proves to be an equally efficient and more economical alternative for successful in vitro micropropagation of olive. The results demonstrate that MLE acts as a natural biostimulant that enhances the morphogenesis of olive micropropagation, largely through its cytokinin (zeatin-type) activity. Its antioxidant constituents may also reduce oxidative stress during culture, potentially lowering the risk of soma clonal variation. Therefore, MLE offers an economical and environmentally friendly approach for the commercial micropropagation of elite olive cultivars.

1. INTRODUCTION

Olive (*Olea europaea L.*) is one of the most important perennial fruit crops cultivated throughout the Mediterranean basin and many subtropical regions because of its exceptional nutritional, economic, and medicinal value. Olive oil is widely recognized as a key component of the Mediterranean diet and is highly valued for its health promoting properties, while table olives represent an important commodity in the global horticultural market(Uylaşer & Yildiz, 2014) Olive cultivation has expanded considerably during the last few decades owing to the increasing demand for high quality olive oil and planting material. The crop occupies approximately 9 million hectares worldwide and contributes substantially to the agricultural economies of many countries (Ahmed et al., 2016). Among commercial cultivars, 'Gemlik' is particularly valued for its superior oil quality and adaptability, making it an important candidate for large scale propagation.

Conventional propagation of olive is mainly achieved through stem cuttings, grafting, and layering. However, the rooting ability of olive is highly genotype dependent, with many commercially important cultivars exhibiting poor rooting capacity, resulting in low multiplication rates and inconsistent nursery

production (Pasha, 2022). Approximately 125 olive genotypes have been classified as difficult to root, highlighting the need for alternative propagation techniques capable of producing uniform planting material efficiently (Micheli et al., 2025). Consequently, in vitro micropropagation has become an important tool for rapid clonal multiplication, germplasm conservation, and the production of disease-free plants (Bayraktar et al., 2020) .

Since the pioneering work of (Rugini, 1984), considerable efforts have been devoted to optimizing olive micropropagation protocols through modifications of basal culture media and plant growth regulator (PGR) combinations. Rugini Olive Medium (OM), Murashige and Skoog (MS) medium, and other modified formulations have been extensively evaluated to improve shoot induction and multiplication efficiency (Murashige & Skoog, 1962; Rugini, 1984). Nevertheless, successful regeneration remains strongly influenced by genotype, physiological condition of the explant, basal medium composition, carbon source, and particularly the type and concentration of plant growth regulators (Bayraktar et al., 2020).

Among plant growth regulators, cytokinins play a central role in regulating cell division, shoot organogenesis, and axillary bud proliferation during in vitro culture. Various cytokinins, including benzyladenine (BA), 2-isopentenyladenine (2iP), and zeatin, have been evaluated for olive micropropagation, however, zeatin consistently produces superior shoot proliferation and shoot quality in many olive cultivars (Rugini, 1984). The effectiveness of zeatin has been attributed to its high biological activity and its ability to stimulate cell division while maintaining normal shoot morphology. Despite these advantages, the commercial application of zeatin is constrained by its high cost, limited stability during sterilization and storage, and increased production expenses associated with commercial scale micropropagation.

Growing environmental concerns and the demand for sustainable horticultural practices have stimulated interest in natural biostimulants as alternatives or supplements to synthetic plant growth regulators. Among these, *Moringa oleifera* Lam. has emerged as one of the most promising plant derived biostimulants because of its exceptional nutritional and phytochemical composition (Grosshagauer et al., 2021). Moringa leaves contain naturally occurring cytokinins, vitamins, amino acids, minerals, antioxidants, phenolic compounds, and soluble sugars that collectively enhance plant growth and physiological performance (Basra & Lovatt, 2016). Moringa leaf extract (MLE) has been reported to improve cell division, chlorophyll synthesis, nutrient uptake, enzymatic activity, and tolerance to abiotic stress in numerous agricultural and horticultural crops (Algopishi et al., 2025; Ali et al., 2018).

In recent years, MLE has also attracted considerable attention in plant tissue culture because of its ability to stimulate morphogenesis and improve regeneration efficiency. The natural cytokinins present in MLE, together with its antioxidant and nutritional constituents, may complement endogenous hormone metabolism and promote shoot proliferation while minimizing oxidative damage commonly observed during in vitro culture of woody species. Previous studies have demonstrated positive effects of MLE on shoot regeneration, biomass accumulation, and plantlet development in several plant species,

suggesting that it may represent an economical and environmentally friendly supplement to conventional tissue culture media (Basra & Lovatt, 2016; Rudiyanto et al., 2023).

Although zeatin is recognized as the most effective cytokinin for olive micropropagation, little information is available regarding its combined use with Moringa leaf extract. Most previous studies have focused on optimizing synthetic plant growth regulators without investigating whether natural biostimulants can enhance cytokinin efficiency or reduce the concentration of expensive growth regulators required for successful regeneration. Evaluating the synergistic interaction between zeatin and MLE may therefore provide a practical approach to improving regeneration efficiency while reducing the overall cost of olive micropropagation.

Zeatin is one of the most effective cytokinins for shoot induction and proliferation in olive tissue culture. However, its use presents several limitations. Zeatin is expensive and thermolabile, and it is prone to degradation in culture media, which reduces its stability and restricts its practical application in large-scale micropropagation. In addition, prolonged or high-dose exposure to synthetic plant growth regulators (PGRs) can induce physiological stress and epigenetic and genetic changes in cultured tissues, increasing the risk of somaclonal variation and compromising the true-to-type nature of regenerated plantlets. These constraints highlight the need for cost-effective and more physiologically compatible alternatives that can reduce dependence on high PGR concentrations. Moringa leaf extract (MLE), a rich source of zeatin-type cytokinins, antioxidants, vitamins, and minerals, may offer such an alternative as a natural supplement to conventional PGRs. However, the effects of MLE applied alone or in combination with zeatin on the in vitro regeneration of olive, particularly the commercially important cultivar 'Gemlik', remain insufficiently understood. Therefore, the present study investigated the individual and combined effects of different concentrations of zeatin and MLE on shoot induction, proliferation, and growth of olive (*Olea europaea* L.) cv. 'Gemlik', with particular emphasis on determining whether MLE can enhance the response to zeatin, allow a reduction in zeatin dose, and support efficient shoot multiplication while minimizing the risk of somaclonal variation.

2. MATERIALS AND METHODS

2.1. Plant Material and Experimental Site

The experiment was conducted during 2025 in the Plant Tissue Culture Laboratory, Agricultural Research Institute (ARI), Tarnab, Peshawar, Pakistan. In vitro-established shoots of olive (*Olea europaea* L.) cv. 'Gemlik' were used as the source of explants. Healthy juvenile nodal segments (1–2 cm long) were excised from actively growing in vitro stock cultures and used for all experiments.

2.2. Preparation of Moringa Leaf Extract

Fresh young leaves of *Moringa oleifera* Lam. were collected and thoroughly washed under running tap water followed by rinsing with sterile distilled water. Thirty grams of fresh leaves were homogenized with 70 mL sterile distilled water using a laboratory blender to obtain a 30% (w/v) stock extract. The

homogenate was filtered through double layer muslin cloth followed by Whatman No. 1 filter paper to remove plant debris. The filtrate was stored in sterile amber bottles at 4°C until use. Working concentrations of 10, 20, and 30 mL L^{-1} were prepared by adding the appropriate volume of stock extract to the culture medium immediately before inoculation.

2.3. Preparation of Zeatin Stock Solution

A trans zeatin stock solution (1 mg mL^{-1}) was prepared by dissolving 10 mg of zeatin powder in a small volume (approximately 200–300 µL) of 0.1 N NaOH. The volume was then adjusted to 10 mL with sterile distilled water. The solution was sterilized by filtration through a 0.22-µm membrane filter and stored in sterile amber vials at 4°C until use. Appropriate volumes of the stock solution were aseptically added to the autoclaved culture medium after cooling to approximately 45°C to obtain final concentrations of 0, 1, 2, and 3 mg L^{-1}.

2.4 Culture Medium and Experimental Treatments

Shoot induction was carried out on Rugini Olive Medium (OM) (Rugini, 1984) supplemented with 30 g L^{-1} sucrose and solidified with 7.5 g L^{-1} agar. The pH of the medium was adjusted to 5.7–5.8 before autoclaving at 121°C and 15 psi for 20 min. Approximately 25 mL of medium was dispensed into each sterile culture vessel.

The experiment consisted of a factorial combination of four zeatin concentrations (0, 1, 2, and 3 mg L^{-1}) and three Moringa leaf extract concentrations (10, 20, and 30 mL L^{-1}), resulting in 12 treatment combinations. The experiment was arranged in a Completely Randomized Design (CRD) with three replications. Each replication consisted of 12 explants, giving a total of 432 explants evaluated during the experiment.

2.5. Explant Inoculation and Culture Conditions

All inoculations were performed aseptically in a laminar airflow cabinet. Forceps, scalpels, and other instruments were sterilized by immersion in 70% ethanol followed by flaming before use. Single node explants were carefully excised and inoculated individually onto the prepared culture medium. Following inoculation, culture vessels were transferred to the growth room and maintained at 22 ± 2°C under a 16-h photoperiod with a light intensity of approximately 40–50 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Scoggins and Bridgen, 2013).

2.6. Data Collection

Observations were recorded throughout the culture period. Days to shoot induction were calculated as the number of days from inoculation to the appearance of visible shoots. Shoot induction percentage was determined using the following equation:

$$Shoot\ Induction\ \% = \frac{Number\ of\ explants\ producing\ shoots}{Total\ number\ of\ explants\ cultured} \times 100$$

At the end of the culture period, the number of shoots per explant, shoot length (cm), and number of leaves per shoot were recorded. Plantlets were gently removed from the culture vessels, washed with distilled water to remove adhering medium, and blotted dry before measuring fresh weight using a digital analytical balance. Dry weight was determined after drying the plantlets at 70°C for 24 h until constant weight (Endeshaw et al., 2015). Relative chlorophyll content was measured using a portable SPAD-502 chlorophyll meter (Konica Minolta, Japan), and the average of two readings per fully expanded leaf was used for statistical analysis.

2.7. Statistical Analysis

The experiment was conducted as a two-factor factorial arrangement in a Completely Randomized Design. Data were subjected to analysis of variance (ANOVA) using STATISTIX version 8.1 (Analytical Software, Tallahassee, FL, USA). Treatment means were separated using the Least Significant Difference (LSD) test at the 1% level of significance, while highly significant differences were interpreted at the 1% probability level following the procedures described by Steel and Torrie (1980).

3. RESULTS

The present study was conducted to evaluate the synergistic effect of varying concentrations of Moringa Leaf Extract (MLE) and Zeatin on the *in vitro* Shoot Induction of Olive cv. 'Gemlik'. The experimental design followed a factorial arrangement under a Completely Randomized Design (CRD). Zeatin and MLE each had a significant effect on all eight Parameters. The Zeatin × MLE interaction was significant for four traits (shoot induction percentage, shoot length, plantlet fresh weight and plantlet dry weight) and non significant for the other four. Means are given in Tables 1–3 (panels A–C). The results regarding shoot induction and morphological development are presented and discussed below.

Days to shoot induction of olive was significantly influenced by the application of Zeatin and MLE levels. While their interaction was found non-significant (Table 1A). Mean data regarding Zeatin concentrations shows that 3 mg L^{-1} Zeatin took the least days (6.09) to shoot induction, which was statistically at par with 2 mg L^{-1} Zeatin (6.12). (Table 1A) indicates the that the least days to shoot induction (7.49) were noted in explants treated with 30 ml L^{-1} MLE, followed by 7.70 days recorded in explants Treated with 20 ml L^{-1} MLE, while maximum days (8.52) to shoot induction were recorded in the treatment with 10 ml L^{-1} MLE.

Results indicate that zeatin and MLE had a significant effect shoot induction as well as by their interaction. Its interaction is given in Fig. 1. The shoot maximum induction percentage (80.91) was observed at 3 mg L^{-1} Zeatin, followed by (80.51) at 2 mg L^{-1} Zeatin), while minimum shoot induction percentage (33.59) was recorded in the explant cultured in media with (0 mg L^{-1} Zeatin).Mean data (Table 1B) indicates that the maximum shoot induction percentage (64.65) were recorded in explants treated with 30 ml L^{-1} MLE. While minimum shoot induction percentage (51.22) were recorded in the treatment with 10 ml L^{-1} MLE.

Table 1
Effect of Zeatin and moringa leaf extract (MLE) on shoot induction of olive cv. 'Gemlik': (A) days to shoot induction and (B) shoot induction percentage. (A) days to shoot induction

Zeatin (mg L⁻¹) MLE 10 ml L⁻¹ MLE 20 ml L⁻¹ MLE 30 ml L⁻¹ Mean				
0	11.40	10.63	10.30	**10.78 A**
1	9.27	8.40	8.20	**8.62 B**
2	7.10	5.70	5.57	**6.12 C**
3	6.30	6.07	5.90	**6.09 C**
Mean	**8.52 A**	**7.70 B**	**7.49 B**	7.90
LSD (P ≤ 0.01): Zeatin = 0.45, MLE = 0.39				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ns (P = 0.114)				
B. Shoot induction (%) (significant interaction)				
0	25.87 i	35.30 g	39.60 f	**33.59 C**
1	32.40 h	41.70 e	47.80 d	**40.63 B**
2	73.10 c	83.03 b	**85.40 a**	**80.51 A**
3	73.50 c	83.43 b	**85.80 a**	**80.91 A**
Mean	**51.22 C**	**60.87 B**	**64.65 A**	58.91
LSD (P ≤ 0.01): Zeatin = 1.07, MLE = 0.93, Zeatin × MLE = 1.85				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ** (P = 0.005)				

*Uppercase letters in Mean rows and columns compare main effects (LSD, P ≤ 0.01), where the interaction was significant, lowercase letters compare the 12 treatment combinations and the best combinations are shaded. ** = significant at P ≤ 0.01, ns = not significant.*

Zeatin and MLE Levels significantly effect the number of shoots explant⁻¹. Their interaction was not significant. The highest Number of shoots explant⁻¹ (2.19) was observed at 3 mg L^{-1} Zeatin, which was statistically at par with 2 mg L^{-1} Zeatin (2.16), while the lowest Number of shoots explant⁻¹ (1.07) was recorded in the explant cultured in media with (0 mg L^{-1} Zeatin). Similarly, the maximum number of shoots per explant (1.83) was recorded in treatments receiving 30 ml L^{-1} MLE, whereas the minimum value (1.72) among the MLE levels was observed at 10 ml L^{-1}.

The data presented in (Table 2B) indicated that shoot length (cm) of olive cultivar was Significantly influenced by different Concentrations of Zeatin, MLE, and its interaction. Among Zeatin levels, the highest mean shoot length (3.25 cm) was obtained at 3 mg L⁻¹ Zeatin, followed closely by 2 mg L⁻¹

Zeatin (3.23 cm), whereas the lowest value (1.15 cm) was recorded in the control treatment. Regarding MLE concentrations, the maximum mean shoot length (2.76 cm) was observed at 30 ml L^{-1} MLE, while the minimum value (2.18 cm) was obtained at 10 ml L^{-1} MLE.

Data with respect to number of leaves $shoot^{-1}$ as affected by various Zeatin and MLE Levels are in (Table 2C). Statistical Analysis prove that Interaction between Zeatins and MLE Levels is not significant. (Table 2C) mean data indicated that the greatest number of leaves $shoot^{-1}$ (10.73) was observed at 3 mg L^{-1} Zeatin, which was statistically at par with 2 mg L^{-1} Zeatin (10.56), while the minimum leaves $shoot^{-1}$ (2.50) was recorded in the explant cultured in media with (0 mg L^{-1} Zeatin).Similarly, leaves $shoot^{-1}$ was significantly affected by MLE concentrations. The highest number of leaves $shoot^{-1}$ (8.43) was recorded in explants treated with 30 ml L^{-1} MLE, followed by 20 ml L^{-1} MLE (7.59) and 10 ml L^{-1} MLE (6.32) respectively.

Table 2
Effect of Zeatin and MLE on shoot growth of olive cv. 'Gemlik': (A) number of shoots explant⁻¹, (B) shoot length and (C) number of leaves shoot⁻¹. A. Number of shoots explant⁻¹

Zeatin (mg L⁻¹)	**MLE 10 ml L⁻¹**	**MLE 20 ml L⁻¹**	**MLE 30 ml L⁻¹**	**Mean**
0	1.02	1.08	1.12	**1.07 C**
1	1.61	1.68	1.75	**1.68 B**
2	2.12	2.15	2.21	**2.16 A**
3	2.14	2.18	2.24	**2.19 A**
Mean	**1.72 C**	**1.77 B**	**1.83 A**	1.77
LSD (P ≤ 0.01): Zeatin = 0.040, MLE = 0.035				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ns (P = 0.802)				
B. Shoot length (cm) (significant interaction)				
0	0.64 i	1.21 h	1.58 g	**1.15 C**
1	1.94 f	2.31 e	2.68 d	**2.31 B**
2	3.06 c	3.23 b	**3.39 a**	**3.23 A**
3	3.09 c	3.26 b	**3.41 a**	**3.25 A**
Mean	**2.18 C**	**2.50 B**	**2.76 A**	2.48
LSD (P ≤ 0.01): Zeatin = 0.053, MLE = 0.046, Zeatin × MLE = 0.092				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ** (P < 0.001)				
C. Number of leaves shoot⁻¹				
0	1.17	2.53	3.80	**2.50 C**
1	4.10	6.30	7.60	**6.00 B**
2	9.90	10.70	11.07	**10.56 A**
3	10.10	10.83	11.27	**10.73 A**
Mean	**6.32 B**	**7.59 A**	**8.43 A**	7.45
LSD (P ≤ 0.01): Zeatin = 1.09, MLE = 0.94				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ns (P = 0.220)				

*Uppercase letters in Mean rows and columns compare main effects (LSD, P ≤ 0.01), where the interaction was significant, lowercase letters compare the 12 treatment combinations and the best combinations are shaded. ** = significant at P ≤ 0.01, ns = not sisignificant*

Fresh weight of olive plantlets was significantly affected by both Zeatin and MLE levels, and its interaction (Zeatin × MLE) was also found to be highly significant shown in Fig. 3. The main effect for Zeatin showed that the highest mean fresh weight (169.96 mg) was observed at 3 mg L^{-1}, while for MLE, the 30 ml L^{-1} concentration resulted in the maximum mean fresh weight (127.26 mg). Mean data in (Table 3A) revealed that the maximum plantlet fresh weight (177.83 mg) was recorded in explants treated with 3 mg L^{-1} Zeatin + 30 ml L^{-1} MLE, which was statistically similar with 2 mg L^{-1} Zeatin + 30 ml L^{-1} MLE (174.20 mg) and 3 mg L^{-1} Zeatin + 20 ml L^{-1} MLE (172.30 mg). These were followed by other treatments involving lower doses of growth regulators, while the minimum plantlet fresh weight (13.13 mg) was recorded in the explant cultured in media with 0 mg L^{-1} Zeatin and 10 ml/ L^{-1} MLE.

(Table 3B) showed that dry plantlets weights of olive cultivar, were significantly affected by media supplemented with different Zeatin and MLE Levels. While the interaction between Zeatin and MLE Levels was also significant shown in Fig. 4. The main effect for Zeatin showed that the highest mean dry weight (41.87 mg) was observed at 3 mg L^{-1}, while for MLE, the 30 ml L^{-1} concentration resulted in the maximum mean fresh weight (33.21 mg). Mean data in Table 3B revealed that the maximum plantlet dry weight (43.87 mg) was recorded in explants treated with 3 mg L^{-1} Zeatin + 30 ml L^{-1} MLE, which was statistically similar with 2 mg L^{-1} Zeatin + 30 ml L^{-1} MLE (42.77 mg) and 3 mg L^{-1} Zeatin + 20 ml L^{-1} MLE (42.20 mg). These results were followed by treatments involving lower concentrations, while the minimum plantlet dry weight (5.10 mg) was obtained in the explant cultured in media with 0 mg L^{-1} Zeatin and 10 ml L^{-1} MLE.

Results indicates that the chlorophyll content was significantly affected by both Zeatin and MLE levels, while their interaction (Zeatin × MLE) was non-significant.Maximum chlorophyll content (51.32) was recorded in explants treated with 30 ml L^{-1} MLE, followed by 20 ml L^{-1} MLE (49.12) and 10 ml L^{-1} MLE (48.29) respectively. Mean data revealed that the chlorophyll content was significantly affected by Zeatin concentrations. The highest SPAD value (49.98) was observed at 3 mg L^{-1} Zeatin, which was statistically at par with 2 mg L^{-1} Zeatin (49.91), while the minimum chlorophyll content (48.51) was recorded in the explant cultured in media with (0 mg L^{-1} Zeatin).

Table 3
Effect of Zeatin and MLE on plantlet biomass and chlorophyll of olive cv. 'Gemlik': (A) fresh weight, (B) dry weight and (C) chlorophyll content (SPAD).

Zeatin (mg L^{-1})	MLE 10 ml L^{-1}	MLE 20 ml L^{-1}	MLE 30 ml L^{-1}	Mean
A. Plantlet fresh weight (mg) (significant interaction)				
0	13.13 g	32.33 f	44.57 f	**30.01 C**
1	68.27 e	95.03 d	112.43 c	**91.91 B**
2	158.77 b	167.80 ab	**174.20 a**	**166.92 A**
3	159.73 b	172.30 ab	**177.83 a**	**169.96 A**
Mean	**99.98 C**	**116.87 B**	**127.26 A**	114.70
LSD (P ≤ 0.01): Zeatin = 7.89, MLE = 6.83, Zeatin × MLE = 13.66*				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ** (P = 0.009)				
B. Plantlet dry weight (mg) (significant interaction)				
0	5.10 h	12.27 g	15.87 f	**11.08 C**
1	20.70 e	26.43 d	30.33 c	**25.82 B**
2	39.53 b	41.20 ab	**42.77 a**	**41.17 A**
3	39.53 b	42.20 ab	**43.87 a**	**41.87 A**
Mean	**26.22 C**	**30.53 B**	**33.21 A**	29.98
LSD (P ≤ 0.01): Zeatin = 1.65, MLE = 1.43, Zeatin × MLE = 2.86				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ** (P < 0.001)				
C. Chlorophyll content (SPAD)				
0	47.50	48.47	49.57	**48.51 B**
1	48.53	49.57	51.60	**49.90 A**
2	48.55	48.90	52.27	**49.91 A**
3	48.57	49.53	51.83	**49.98 A**
Mean	**48.29 B**	**49.12 B**	**51.32 A**	49.57
LSD (P ≤ 0.01): Zeatin = 1.10, MLE = 0.95				
ANOVA: Zeatin **, MLE **, Zeatin × MLE ns (P = 0.417)				

Uppercase letters in Mean rows and columns compare main effects (LSD, P ≤ 0.01), where the interaction was significant, lowercase letters compare the 12 treatment combinations ** = significant at

P ≤ 0.01, ns = not significant. *Interaction LSD for fresh weight recalculated from the error mean square (35.775, 24 df), the thesis lists 6.66.

4. DISCUSSION

4.1. Zeatin was the main driver of the response

The present study demonstrated that Zeatin had a significant effect on all in vitro growth and regeneration parameter of olive cultivar with clear differences observed between highest and lowest responses across treatments. This agrees with the role of cytokinins as the main regulators of cell division, differentiation and shoot organogenesis in culture (Trottet et al., 2022). In olive, which has strong apical dominance and naturally limited branching (Jiménez-Ruiz et al., 2015), an external cytokinin supply is what releases axillary buds. (Chamandoosti, 2025) reported faster and more frequent shoot induction with Zeatin in olive, and(Allatif & Hmmam, 2022) found that cytokinin-poor media slowed shoot initiation and lowered regeneration. The Zeatin-free explants in our study were the slowest and poorest on every measure, which fits these reports. Zeatin at 2 and 3 mg L^{-1} was statistically equal for every trait. The response therefore appears to level off between 1 and 2 mg L^{-1}, and raising the dose to 3 mg L^{-1} gave no measurable gain.(Micheli et al., 2025) found that ‘Moraiolo’ olive multiplied best at 2 and 4 mg L^{-1} Zeatin, and(Králová et al., 2024) linked higher cytokinin levels to stronger proliferation in olive cultures, so the optimum seems to lie within this broad range. Because 3 mg L^{-1} was the highest dose we tested, we cannot say whether a higher dose would help or harm. The multiplication rate was also modest, with at most 2.24 shoots per explant. That is expected for single-node explants after one culture cycle, but it is lower than the multiplication rates cited for other cultivars, which(Debnath et al., 2021; Kaur & Sharma, 2009) show depend strongly on cultivar and medium.

4.2. MLE acted as a supplementary source of growth promoters

The findings of the present study indicated that Moringa Leaf Extract (MLE) had a clear and meaningful impact on the in vitro growth and regeneration of olive cultivar, with distinct differences between higher and lower concentration treatments. Moringa leaves contain zeatin-like cytokinins, vitamins, minerals, amino acids and antioxidants (Basra & Lovatt, 2016; Fahey, 2005; Makkar & Becker, 1996), and the extract has acted as a biostimulant in many crops (Arif et al., 2023).(Nihayati & Najah, 2021) compared MLE and Zeatin directly on in vitro regeneration of bud explants and found that the extract was effective. Our results extend that finding to olive. Most traits increased steadily up to 30 ml L^{-1} without levelling off, so the optimum MLE level may be above the range we tested. We did not measure the hormone content of the extract. Whether the effect came from its cytokinin-like compounds, its minerals and antioxidants, or both therefore remains an open question.

4.3. The Zeatin × MLE interaction

The interaction was significant for shoot induction percentage, shoot length, fresh weight and dry weight. In all four, the benefit of MLE shrank as the Zeatin dose increased. Raising MLE from 10 to 30 ml L^{-1} increased shoot induction by 53% without Zeatin but by only 17% at 3 mg L^{-1}. For shoot length the gain was 147% against 10%, for fresh weight 3.4-fold against 11%, and for dry weight 3.1-fold against 11%. This is a pattern of diminishing returns, not of synergy. The effect of Zeatin was also smaller at high MLE than at low MLE (shoot length, for example, rose by 2.45 cm from 0 to 3 mg L^{-1} at 10 ml L^{-1} MLE but only 1.83 cm at 30 ml L^{-1}). The combined response was therefore less than the sum of the two separate responses. A likely explanation is that the cytokinin-like activity of MLE(Nihayati & Najah, 2021) overlaps with that of Zeatin. MLE then matters most when Zeatin is scarce, and adds little once Zeatin has saturated the response. The thesis hypothesis of synergy is therefore only partly supported: the combination was clearly better than either factor alone, but the extra benefit was not additive. The interaction was also small in absolute terms for induction percentage, accounting for less than 0.1% of the total sum of squares. For days to shoot induction, shoot number, leaf number and chlorophyll content the interaction was not significant, so the two factors acted independently on those traits.

The same data support a practical conclusion. Zeatin at 2 mg L^{-1} with 30 ml L^{-1} MLE was statistically equal to 3 mg L^{-1} with 30 ml L^{-1} for induction percentage (85.40% against 85.80%), shoot length (3.39 against 3.41 cm), fresh weight (174.20 against 177.83 mg) and dry weight (42.77 against 43.87 mg). Zeatin is costly and unstable, which is the premise of this study, and Pannelli et al. (2023) have likewise explored a natural additive to reduce its use in olive micropropagation. A third of the Zeatin can thus be saved without a measurable loss. MLE could not replace Zeatin, however: the Zeatin-free medium with 30 ml L^{-1} MLE reached only 39.60% induction and 1.58 cm shoot length. Lowering Zeatin below 2 mg L^{-1} was also not supported, since 1 mg L^{-1} with 30 ml L^{-1} MLE gave only 47.80% induction.

4.4. Shoot growth and biomass

The Zeatin effect on shoot length, leaf number and plantlet weight is consistent with cytokinin-driven cell division and organ initiation reported for olive and other woody species(Allatif & Hmmam, 2022b; Benelli & De Carlo, 2018; Micheli et al., 2025; Šmeringai et al., 2023) reported higher biomass in plantlets grown with cytokinin, which matches the 13-fold difference in fresh weight between our control (13.13 mg) and the best combination (177.83 mg). MLE has also raised fresh and dry biomass in several crops (Abd El-Mageed et al., 2017; Abdelkhalik et al., 2025; Nouman et al., 2013). The strong interaction for these traits arises mainly because the Zeatin-free plantlets were so small that they had the most room to respond to MLE. Once plantlets reached about 160 mg of fresh weight at 2 to 3 mg L^{-1} Zeatin, MLE added comparatively little.

4.5. Chlorophyll content

Chlorophyll was the one trait where MLE mattered more than Zeatin (about 63% against 15% of the total sum of squares). Cytokinins delay chlorophyll breakdown and support chloroplast development(Werner et al., n.d.; Akhtar et al., 2020; El-Lethy et al., 2024; Hörtensteiner & Kräutler, 2011; Schelbert et al., 2009; Wang et al., 2020) which may explain why all Zeatin levels exceeded the control. MLE has raised

chlorophyll content in other crops (Nouman et al., 2013; Yasmeen et al., 2013), Probably through its supply of minerals and antioxidants as well as its hormones. The differences were small, however, with a total spread of about 3 SPAD units (48.29 to 51.32), so their practical value is limited. The lack of interaction suggests that the two factors raised chlorophyll through separate routes.

5. CONCLUSION

This study shows that zeatin and moringa leaf extract (MLE) both improve the in vitro shoot induction and growth of olive cv. 'Gemlik', and that they work better together than alone. Zeatin was the dominant factor. Compared with the zeatin-free, 2 and 3 mg L^{-1} shortened the time to shoot induction from 10.78 to about 6.1 days. MLE gave a smaller but consistent benefit to all eight traits, with the best results at 30 ml L^{-1}.The Zeatin × MLE interaction was significant for shoot induction percentage, shoot length, and plantlet fresh and dry weight. The highest values occurred with 3 mg L^{-1} Zeatin + 30 ml L^{-1} MLE (85.80% induction, 3.41 cm shoots, 177.83 mg fresh weight and 43.87 mg dry weight). The combination of 2 mg L^{-1} Zeatin + 30 ml L^{-1} MLE was statitically equal for induction percentage (85.40%), shoot length (3.39 cm), fresh weight (174.20 mg) and dry weight (42.77 mg). MLE raised performance most when zeatin was low and added less once zeatin was sufficient. MLE alone could not replace zeatin: with 30 ml L^{-1} MLE and no zeatin, induction reached only 39.60%. For days to shoot induction, shoot number, leaf number and chlorophyll content, the two factors acted independently.

For efficient and cost-effective shoot induction of 'Gemlik', use 2 mg L^{-1} Zeatin with 30 ml L^{-1} MLE. This gives results equal to the highest zeatin dose while using one third less of the costly hormone.

Further studies should optimize even lower Zeatin concentrations with MLE for large-scale propagation of olive Cv.Gemlik and other cultivars.

Declarations

Author Contribution

Rovaid Ali conceived and designed the study, conducted the experiments, collected and analyzed the data, and wrote the original manuscript. Naveed Ahmad and Ghani Gul supervised the research, contributed to the interpretation of the results, and critically reviewed and revised the manuscript. Both authors read and approved the final manuscript.

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Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

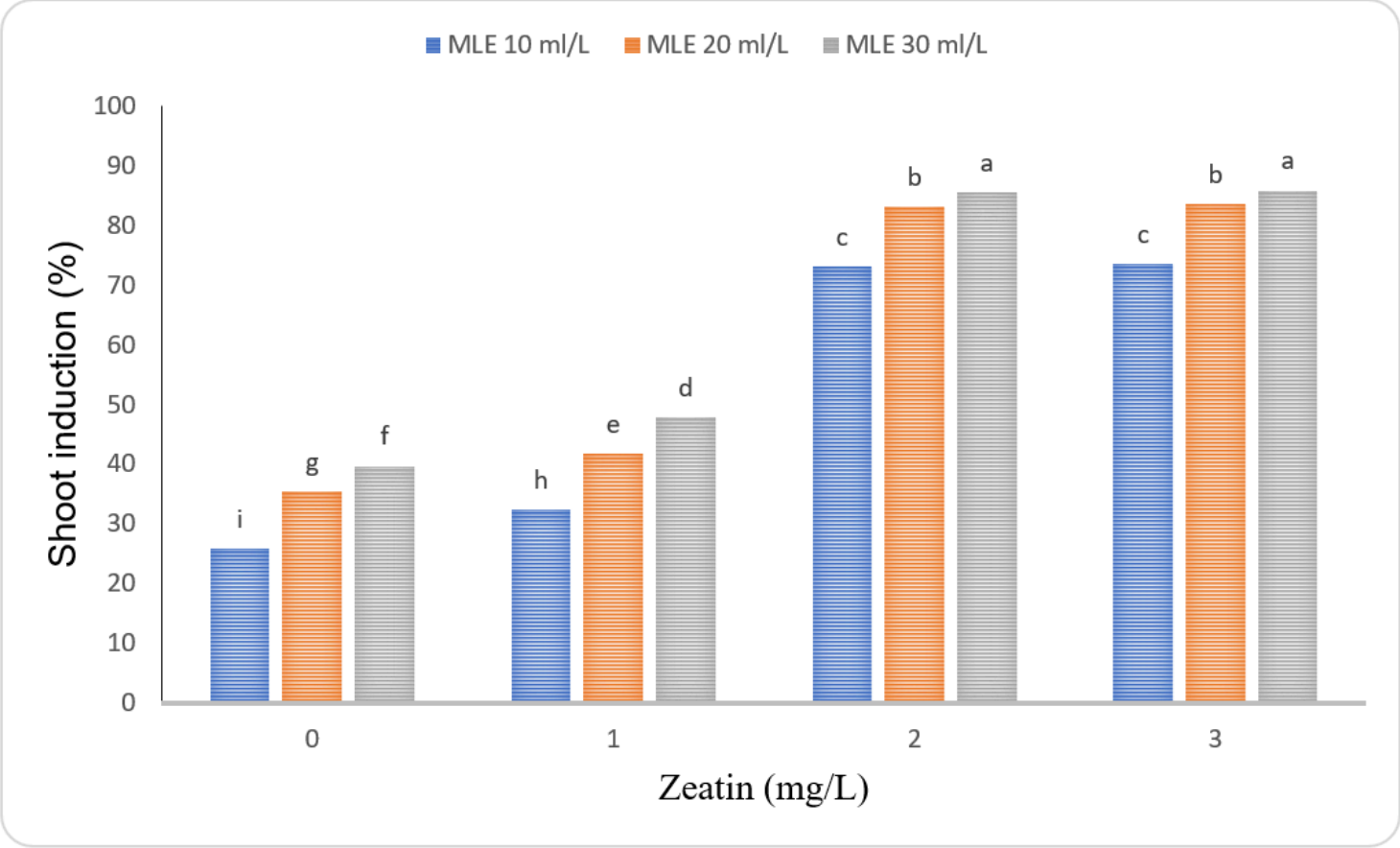


Figure 1

Interaction effect of Zeatin and MLE on shoot induction percentage of olive cv. ‘Gemlik’. Bars sharing the same letter do not differ significantly (LSD, P ≤ 0.01).

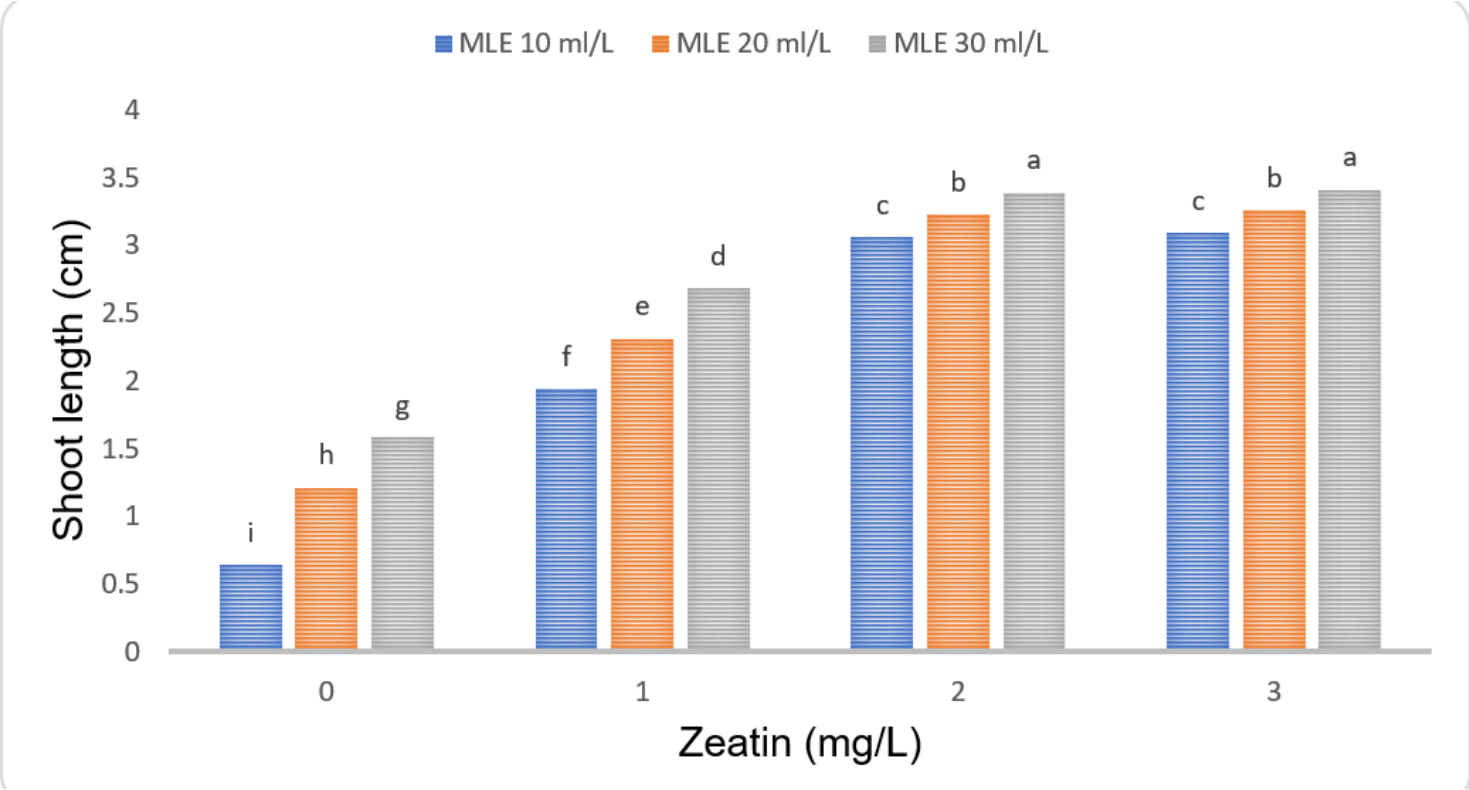


Figure 2

Interaction effect of Zeatin and MLE on shoot length (cm) of olive cv. ‘Gemlik’. Bars sharing the same letter do not differ significantly (LSD, P ≤ 0.01).

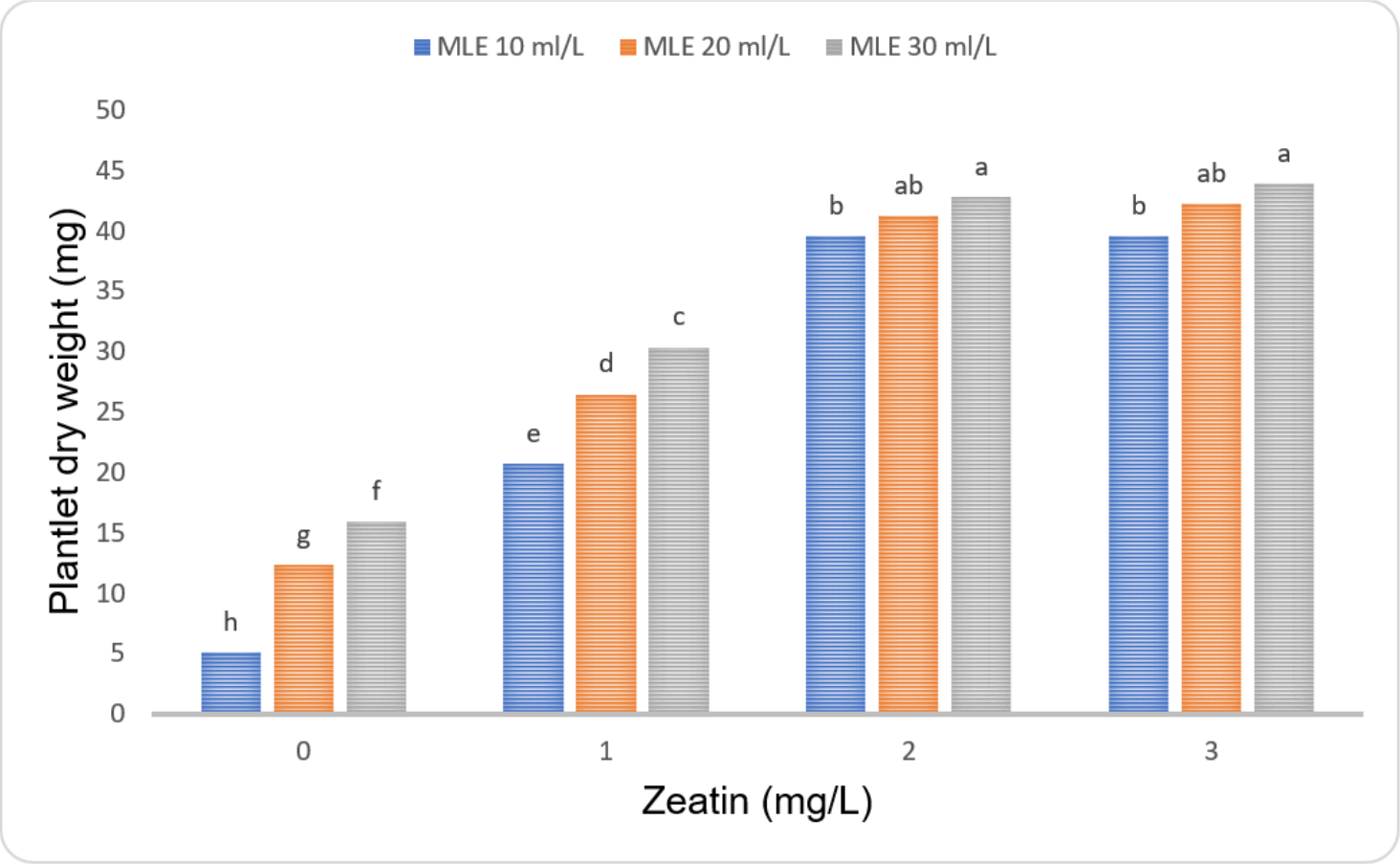


Figure 3

Interaction effect of Zeatin and MLE on plantlet fresh weight (mg) of olive cv. ‘Gemlik’. Bars sharing the same letter do not differ significantly (LSD, P ≤ 0.01).

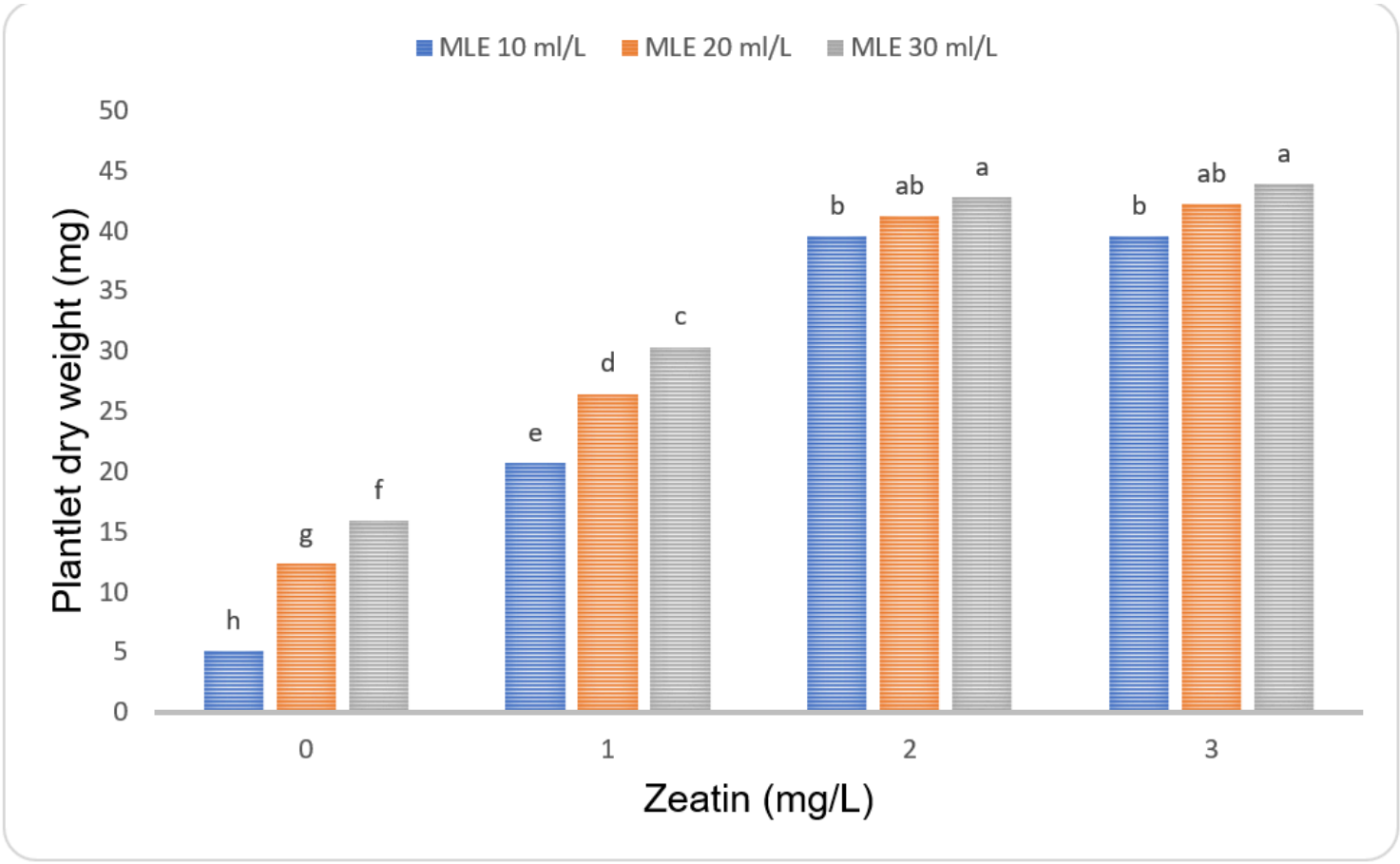


Figure 4

Interaction effect of Zeatin and MLE on plantlet dry weight (mg) of olive cv. ‘Gemlik’. Bars sharing the same letter do not differ significantly (LSD, P ≤ 0.01).