Research article

Investigating the impact of paclobutrazol and tannic acid on floral development of *in vitro*-grown cannabis plantlets

Ladan Ajdanian [a,b,c,d,e], Hossein Arouiee [a,*], Andrew Maxwell Phineas Jones [f], Mohsen Hesami [f], Hossein Nemati [a], Marco Pepe [f]

[a] *Department of Horticultural Sciences and Landscape Architecture, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[b] *Département de phytologie, Université Laval, Québec City, QC, Canada*
[c] *Institut de Biologie Intégrative et des Systèmes (IBIS), Université Laval, Québec City, QC, Canada*
[d] *Centre de recherche et d'innovation sur les végétaux (CRIV), Université Laval, Québec City, QC, Canada*
[e] *Institute Intelligence and Data (IID), Université Laval, Québec City, QC, Canada*
[f] *Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada*

A R T I C L E   I N F O

A B S T R A C T

Gibberellic acid (GA_3) is inhibitory to floral development of *in vitro* cannabis plants and inhibiting GA_3 biosynthesis promotes floral development. As such, paclobutrazol (PBZ), a potent GA_3 biosynthesis inhibitor may be useful for increasing floral biomass and expediting development, but due to health concerns, its use is prohibited in cannabis production. The present study was conducted to compare the use of PBZ with tannic acid (TA), a natural compound with potential GA_3 inhibiting characteristics. Results confirmed that PBZ significantly affected the number of flowers, percentage of flowering plantlet, and flower appearance time. Treatment using PBZ at a concentration of 10 μM resulted in the greatest number of flowers (7.95) compared to other treatments. Moreover, this compound at concentrations of 5 and 10 μM yielded the highest percentage of flowering plantlets, at 75 % and 70 %, respectively. Flowers also appeared 7–15 days sooner than other treatments. Additionally, the energy transfer efficiency in the photosynthetic system and chlorophyll concentration in plants treated with PBZ were considerably higher than those under other treatments. Under the PBZ treatment, the length of internode was significantly reduced. In contrast, TA generally had the opposite effect of PBZ, suggesting that it does not act as a GA_3 inhibitor in this context. Furthermore, positive effects of TA at a concentration of 10 μM were observed on total leaf area (840.08 mm^2) and stem length (40.09 mm). The highest number of leaves (12.5) was found in the presence of TA at a concentration of 100 μM. TA at its highest concentration (1000 μM) had an inverse effect on cannabis growth and flowering but was likely due to toxicity rather than any inhibitory effects. Consequently, the obtained results confirm the importance of growth regulators and natural compounds on plant growth and can broaden our understanding for future research and achievement of objectives.

1. Introduction

Cannabis sativa is a member of the Cannabaceae family that is cultivated in many parts of the world for a wide variety of

---

* Corresponding author.
  E-mail address: aroiee@um.ac.ir (H. Arouiee).

applications such as producing oil, fiber, seeds, and its medicinal properties [1,2]. Accordingly, an exponential rise in demand for cannabis products can be seen across the globe [3]. Nonetheless, the long history of the prohibition of cannabis cultivation has restricted research on this multi-purpose species and biotechnological methods in this area are still in their early stages [4]. However, the past decade has witnessed a significant increase in cannabis tissue culture research, which is confirmed by the high number of academic publications on this topic [5–7]. Although, it should be noted that considerable challenges still remain with respect to studies on tissue culture of cannabis [6,8].

Tissue culture is a simple but effective technique for growing and manipulating plants in a controlled and aseptic environment. As a result, it is a very useful and practical method for understanding various aspects of developmental biology and improving plant breeding in many species [9]. This technique offers several advantages, including reduced space requirements, sterile cultivation in preventing pests, diseases, and pathogens transmission, and highly efficient production and storage of mature plants safe from weather, pests, and disease [10]. From a breeding perspective, in some species *in vitro* techniques can be used to reduce generation time, thereby increasing genetic gains over time. However, *in vitro* breeding strategies can be complicated by challenges in the induction and development of flowers *in vitro*. While some species flower readily *in vitro*, others do not and some reports of a decrease in the quality of flowers grown in *in vitro* have been reported and *in vitro* breeding is not always possible [11]. In the case of cannabis, flowers develop in response to short days in a tissue culture environment [12] as would be expected based on standard growth responses for the species, making the possibility of *in vitro* breeding realistic. Additionally, flowering in cannabis can generally be classified into three groups: early, medium, and late flowering [13]. However, the developing inflorescences tended to be less robust than those produced in a normal setting and their fertility and ability to produce seed are unknown.

Since plant breeding programs are considered long, complicated processes, it generally requires several years for desirable outcomes to be achieved. Due to the iterative process, shortening the time required for each cycle can have a significant impact on the rate of genetic gains. This issue is economically significant as a shorter time required for the plant breeding program means reduced production costs which leads to a reduction in the price of the final products for consumers [14]. Accordingly, it is essential to shorten the production period while accelerating breeding programs and raising the level of precision, to increase the efficiency of plant products. One of the strategies proposed for shortening growth cycles is using *in vitro* culture techniques [15]. It is a suitable strategy for growing plants in artificial environments and providing conditions optimized for flower generation and growth in plants. Given the recent reports, several successful *in vitro* protocols have been proposed by breeders, for plants such as pea (*Pisum sativum* L.) [16], lentil (*Lens culinaris* Medikus.) [17], chickpea (*Cicer arietinum* L.) [18], bean (*Phaseolus vulgaris* L.) [19], and broad bean (*Vicia faba* L.) [20].

By managing and optimizing these factors in line with increasing plant growth, researchers can alter the period required for plant growth in each stage (vegetative or reproductive). As chemical messengers, plant growth regulators play an important role in driving plant growth which can affect different aspects of growth including cell division, elongation, and differentiation [21]. Several growth regulators, inhibitors, and additives have been introduced that significantly affect plant growth. For instance, triazoles are a class of inhibitor are regarded as protective against plant stress, due to their innate ability to induce abiotic stress tolerance through increasing enzymes and antioxidants in plants experiencing stress [22]. Specifically, paclobutrazol (PBZ) is an inhibitor of GA_3 biosynthesis, which prevents the oxidation of ent-kaurene into ent-kauronoic acid through deactivating the oxygenase associated with P450 cytochrome [23,24]. It is also reported that PBZ affects the synthesis and natural catabolism of abscisic acid [25]. Ultimately, the effects of PBZ on both synthesis and catabolism processes result in an increase in the concentration of abscisic acid in leaves.

GA_3 has been documented to impact floral development in plants, but the effects are highly species specific [26]. For example, application of GA_3 can either inhibit or promote flowering depending on the plant species, as well as impacting sex determination of flowers in some monoecious and dioecious plants [27]. In the case of cannabis, GA_3 has been found to induce male flower development in genetically female plants [28]. Further, the GA_3 biosynthesis inhibitor PBZ, has been found in illicit cannabis samples where it is used to promote earlier and heavier flowering [29]. Subsequent research studying *in vitro* flower development has verified that GA_3 inhibits flower development in cannabis while PBZ promoted flowering [30].

Tannins are a class of polyphenols that occur in nature and can easily be extracted from plants [31]. Tannic acid (TA) is a natural tannin and a derivative of phenolics belonging to the gallotannin class of compounds [32]. This organic acid can be extensively found in plants, such as in the leaves and skins of many species such as tea (*Camellia sinensis* L.) [33]. Tannins are generally thought to inhibit plant growth enzymes and proteins [34]. In relation to this inhibitory role, tannin's non-specific binding with GA_3 or some GA_3-stimulated growth mediators has been reported [34]. It has also been reported that GA_3 or the system on which GA_3 operates is very sensitive to tannins. Remarkably, at high concentrations they can inhibit gibberellin activity and prevent growth, but in low concentrations they can have the opposite effect [35]. Based on these observations, tannins may represent a less toxic natural alternative to PBZ that can manipulate the GA_3 response in cannabis to impact floral development.

The present study was conducted to compare the effects of PBZ and TA on *in vitro* flower development in cannabis to evaluate the potential of TA as an alternative to PBZ. To this aim, tissue culture was employed to enhance cannabis growth and reduce its growth cycle, while producing flowers in high percentages. This was done to find the best treatment for *in vitro* growth optimization and flower generation of cannabis. Both the chemical and the natural compounds were expected to contribute to achieving reduced growth cycle and flower appearance in the shortest possible time; a comparison between these compounds was carried out throughout this growth process.

2. Materials and method

2.1. Plant materials, culture medium, and growth conditions

A clonal cultivar of *Cannabis sativa* L. cv. Roto, established and maintained in tissue culture was used for this study. The plants considered in this experiment were subcultured using nodal explants; each subsample included three nodes with no leaves. Effort was taken to select uniform explants from the middle nodes of the plants. The culture medium used was composed of full strength DKW salts and vitamins (Phytotech, Kansas, US), 3 % sucrose, 0.6 % agar (Thermo-Fisher Scientific, Waltham, MA), and a pH of 5.8 [36]. The treatments used in this experiment included TA as a natural compound and PBZ as the chemical compound (Phytotech, Kansas, USA). In addition to using a control treatment, TA was employed at three levels of 10, 100, and 1000 μM, while PBZ was used at two levels of 5 and 10 μM. TA and PBZ's molecular masses were used to create the stock solutions (water solvent). Equal volumes of both stock solutions were prepared to a volume of 300 mL and prepared to different concentrations for both treatments. TA, unlike PBZ, cannot be autoclaved due to its heat sensitivity, so it was filter sterilized and added to the media after it was autoclaved and cooled to approximately 60°. Forty ml of each media was placed in each Magenta GA7 vessel (Fisher Scientific, NJ, USA). Each vessel contained four explants; For vegetative growth (two week), they were illuminated using full spectrum LED lighting (Intravision Spectra Blades, including 75 % Red (R) (600–700 nm), 19 % Green (G) (500–600 nm) and 6 % Blue (B) (400–500 nm)) with a long photoperiod of 16:8 (light:dark) and an intensity of 25 μmol/m^2/s, within the growth chamber with a temperature of $25 \pm 2°C$ (Fig. S1a). After two weeks, to induce flowering (five weeks), the plants were illuminated via a mixture of 75 % R (600–700 nm), 12.5 % B (400–500 nm), and 12.5 % White (400–700 nm) lights with a photoperiod of 12:12 [12] (light: dark) and an intensity of 100 ± 10 μmol/m^2/s (Fig. S1b).

2.2. Data collection and experimental design

The time of the appearance of the first flower was evaluated through daily visual inspections of all plants. We did assess flowering at a stage where plants were fully developed and ready for pollination. After 50 days, the plants were taken out of the vessels for final data collection. Morphological traits collected included the lengths of root and stem, number of leaves and nodes, length of internode, percentage of flowering plantlet, the number of flowers per plant, the fresh weight of the plantlet (shoot and root part), and the total leaf area. All the plants in the vessel were removed and placed on sterile paper for measurements. The roots were separated from the stems. The leaves were detached and counted. Additionally, the number of nodes on the stem was counted. To count the female flowers in the cannabis plant, the number of flowers in each inflorescence was carefully counted and then the total number was calculated for the whole plant. Female flowers were identified using maturity criteria, such as emergence of styles and full flower development.

Image processing techniques were used to measure the lengths of stem and root, length of internode and the total leaf area. Photos of the grown plantlets were captured using an iPhone 13, capable of autofocus from a vertical distance of 25 cm. It should be noted that different studies have confirmed the reliability of cellphones in capturing images for examining morphological traits in plants [37–40]. Next, the captured photos were processed using ImageJ I.50i Software (National Institute of Mental Health). The image processing steps were as follows: (a) setting the scale and acquiring the image; (b) RGB (red, green, and blue) color cropping and filtering; (c) producing a grayscale image; (d) developing a binary image; (e) eliminating noise from the binary image and filling in any gaps; and (f) measuring the traits. Specifically, for total leaf area analysis, first, the leaves were detached and then arranged flat on sterile paper before photographing.

The CCM-300 (Hoskin, Canada) device was used to examine photosynthetic traits including the efficiency of energy transfer in the photosynthetic system or CFR (Chlorophyll-Fluorescence Ratio F735/F700) and chlorophyll concentration (mg/m^2). For this purpose, we selected three fresh green leaves from different parts of the stem (top, middle, and bottom).

2.3. Statistical analyses

The experiment was conducted based on a Complete Randomized Design (CRD) involving five replicates and four observations per replicate. In addition, trait analysis (ANOVA) and mean comparison (the LSD test) ($p \leq 0.05, 0.01$) were carried out using the All-in-One package [41] in the R Software (Ver. 4.3.1) and the SAS Software (Ver. 9.3). The mean comparison charts were drawn using Microsoft Excel.

3. Results

3.1. Effects of TA and PBZ on growth and flowering

Examinations and analysis of variance of the data collected in this study showed that the application of PBZ and TA in parameters including the number of nodes and leaves, percentage of flowering plantlet, stem length, length of internode, total leaf area, chlorophyll concentration and the CFR were significant ($p \leq 0.01$). Moreover, parameters such as the number of flowers and the shoot fresh weight were significant ($p \leq 0.05$). However, neither compound had a significant effect on root length or Total Fresh Weight and its root.

The mean comparison chart (Fig. S2a) demonstrates the lowest number of nodes (3) on the TA treatment at its highest concentration (1000 μM), however, this appeared to be due to toxic effects of TA rather than an inhibitory response based on the overall reduction of growth. Regarding the number of leaves (Fig. S2b), the obtained results showed that the lowest number (2.75) belonged to

the TA treatment at a concentration of 1000 μM; the highest number of leaves (12.5) also resulted from the TA treatment, albeit at a concentration of 10 μM.

The highest number of flowers was observed in PBZ treatment at a concentration of 10 μM which, compared to the control treatment, showed a 59.74 % increase (Fig. 1a). Also, the highest percentage of flowering plantlet was observed in PBZ treatment at concentrations of 5 and 10 μM, with 80 % and 75 %, respectively (Fig. 1b). In contrast, increasing TA concentrations up to 100 μM decreased the number of flowers. In other words, the application of TA at high concentrations has no positive effects on percentage of plants flowering since the lowest percentage of flowering plantlet (10 %) belonged to TA treatment at concentrations of 100 and 1000 μM (Fig. 1b).

The results of the mean comparison did not show any significant difference between treatments for shoot fresh weight, total plantlet, root, and root length (Fig. S3).

The highest stem length (40.09 mm) was observed in TA treatment at a concentration of 10 μM; however, in the TA concentration of 1000 μM, a 69 % reduction in stem length was seen (Fig. 2a). The longest length of internode recorded (5.84 mm) was found in the plantlet treated via TA at a concentration of 10 μM, which was a 38.69 % increase compared to the control treatment. Meanwhile, the application of PBZ (10 μM) led to the shortest length between nodes (1.2 mm) (Fig. 2b).

According to the results of mean comparison, the highest total leaf area (840.08 mm^2) was observed in the TA treatment (10 μM). Moreover, the lowest one was found in TA treatment (1000 μM), as 31.77 mm^2 (Fig. 3a).

The highest energy transfer efficiency (CFR) in the photosynthetic system was found to be 1.41, in PBZ treatment applied at two concentration levels (Fig. 3b); however, in TA at (100 μM), this value reduced by 24.64 %. In addition, no significant differences were observed between the control treatment and TA at 10 μM (The TA treatment at a concentration of 1000 μM was removed from this analysis, as the number of leaves in the plant was not sufficient for analysis using the intended device).

The highest chlorophyll concentration was obtained in the PBZ (5 μM) treatment as 506.35 mg/m^2, which was significantly different from the control treatment. In addition, this value reduced by 41 % in the TA (100 μM) (Fig. 3c) (The TA1000 treatment was removed from this analysis, as the number of leaves in the examined plant was not sufficient for conducting analysis using the intended device).

To examine the process of the onset of flowering, percentage of flowering plantlet (the number of plants in which flowers were observed) were monitored periodically (Fig. 4). The trend chart showed that flowers appeared in plants in the PBZ treatment when the photoperiod (12:12) changed, specifically, on the 21st day following subculture; this was 1–2 weeks earlier compared to plants in other treatments. By the end of the 4th week that is, the 28th day, the first flower was seen in cannabis plantlets in the TA treatment at its lowest concentration (10 μM); accordingly, raising the concentration of treatment led to a one-week delay in flower appearance. Additionally, no differences were observed between the control treatment and TA at a concentration 10 μM, in terms of the flowering time. Fig. 5(a–f) represents an image of plants affected by each treatment at the end of the experiment (day 50). Additionally, the plantlet configuration has been included in the supplementary material (Fig. S4).

3.2. Correlation between the traits in TA and PBZ treatments

This section presents an examination of correlation results between different parameters. As can be seen in Fig. 6, a positive and significant correlation (r = 0.96) at 1 % probability level was observed between the number of nodes and the number of leaves. There was also a positive relation between the number of nodes, and root length and CFR value with correlation coefficients of 0.93 and 0.92 (p ≤ 0.01), respectively. The number of nodes also had a positive relation with total leaf area (r = 0.90) and chlorophyll concentration (r = 0.84) (p ≤ 0.05). The number of leaves parameter had a positive correlation with other traits; however, such a correlation was not found to be significant. For example, the lowest correlation value (r = 0.11) was found between the number of nodes and the length of internode.

A positive and significant correlation was found between the number of leaves and root length (r = 0.91). In other words, the
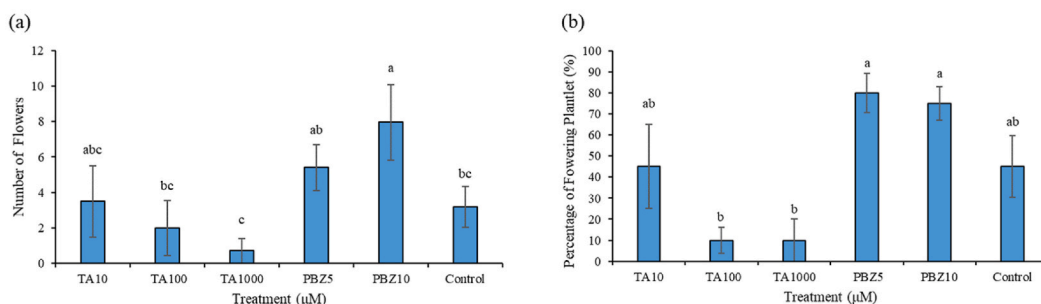

Fig. 1. Number of Flowers (a) and percentage of flowering plantlet (b) Mean comparison (LSD, P ≤ 0.05 and 0.01 respectively) of *in vitro Cannabis sativa* L. cv. Roto after application of TA and PBZ treatments. Different letters indicate significant differences between treatments by LSD test. (TA10): Tannic Acid 10 μM, (TA100): Tannic Acid 100 μM, (TA1000): Tannic Acid 1000 μM, (PBZ5): Paclobutrazol 5 μM, (PBZ10): Paclobutrazol 10 μM.

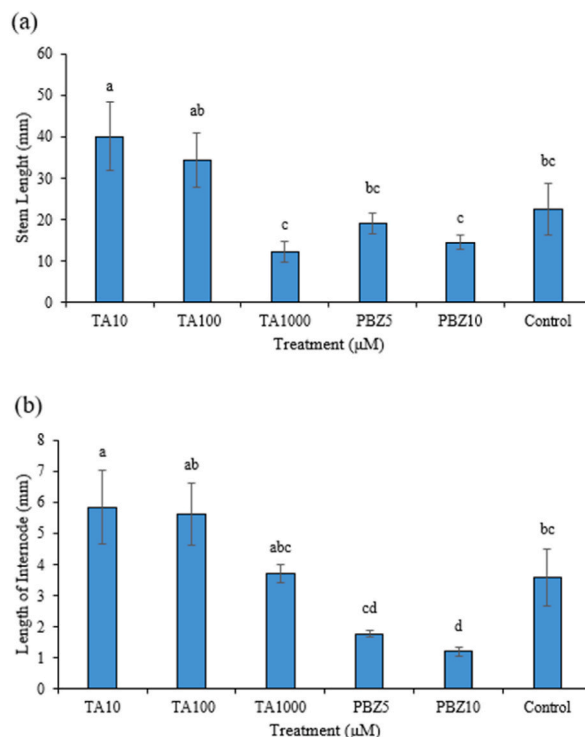**Fig. 2.** Stem Length (a) and length of Internode (b) Mean comparison (LSD, P ≤ 0.01) of *in vitro Cannabis sativa* L. cv. Roto after application of TA and PBZ treatments. Different letters indicate significant differences between treatments by LSD test. (TA10): Tannic Acid 10 μM, (TA100): Tannic Acid 100 μM, (TA1000): Tannic Acid 1000 μM, (PBZ5): Paclobutrazol 5 μM, (PBZ10): Paclobutrazol 10 μM.

increase in root length led to an increase in the number of leaves. The number of leaves was found to have positive correlations with other traits as well. The number of flowers had no significant relations with any of the other traits; its only positive correlation pertained to the chlorophyll concentration value ($r = 0.81$), at 5 % probability level. Conversely, the number of flowers was found to have negative correlations with stem length and length of internode; and it had a positive correlation with total leaf area with a low coefficient ($r = 0.020$). A positive and significant correlation ($r = 0.83$) was observed between stem length and total leaf area. Root length was found to be positively and significantly correlated with total leaf area ($r = 0.96$) and CFR value ($r = 0.85$). The parameter of length of internode was found to have a negative and insignificant relation with CFR and the concentration of chlorophyll in cannabis plantlets. Furthermore, the CFR value showed a positive, significant correlation with chlorophyll concentration ($r = 0.98$).

4. Discussion

The main purpose behind examining TA and PBZ, was to compare their effects on optimizing the growth conditions of *in vitro* cannabis plantlets and gain deeper insight into the developmental biology of cannabis. Results confirmed previous studies that showed that PBZ considerably affected parameters such as increasing the number of flowers, percentage of flowering plantlet, and the appearance time of flowers [30]. The onset of the florigenic stage and the appearance of flowers are considered important stages in a plant's life cycle, and the timing of these stages is of utmost importance. Studies conducted with a focus on physiological and genetic aspects demonstrate the complexity of mechanisms involved in these transitions, and the involvement of changes in apical meristem from the vegetative stage into the reproductive stage [42]. The ability of explants to flower *in vitro* and the quality of the flowers produced, depends on a variety of factors such as chemical and natural materials, growth regulators, and other unknown factors [43].

Of the common plant growth regulators used in plant cultivation, growth inhibitors, particularly PBZ, limit vegetative growth and subsequently induce flowering in several species, as example mango (*Mangifera indica* L.) *in vivo* condition [44], spraying PBZ on *Lilium* [45], peach (*Prunus Persica* L.) [46], apple (*Malus domestica* L.), and pear (*Pyrus communis* L.) [47]. Notably, PBZ reduces vegetative growth speed by blocking growth at early stages. In turn, this leads to the accumulation of carbohydrates and a reduction in the total amount of nitrogen in the stem. It also contributes to flowering in plants by altering the C:N ratio [48]. Important factors in flowering induction include photosynthetic material input, energy flow, and the distribution of absorbed materials. Therefore, the application of PBZ leads to the onset of flowering in some plants by reducing the gibberellin levels while raising the levels of auxins and cytokinins at the tip of aerial organs [49]. Since plant growth is not regulated through a single, specific phytohormone, responses to PBZ stimulus in flowering induction may also depend on its effects on growth regulators other than gibberellins. There are reports in this regard in which the isoprenoid pathway related to gibberellin biosynthesis regulate the biosynthesis pathway of other phytohormones such as
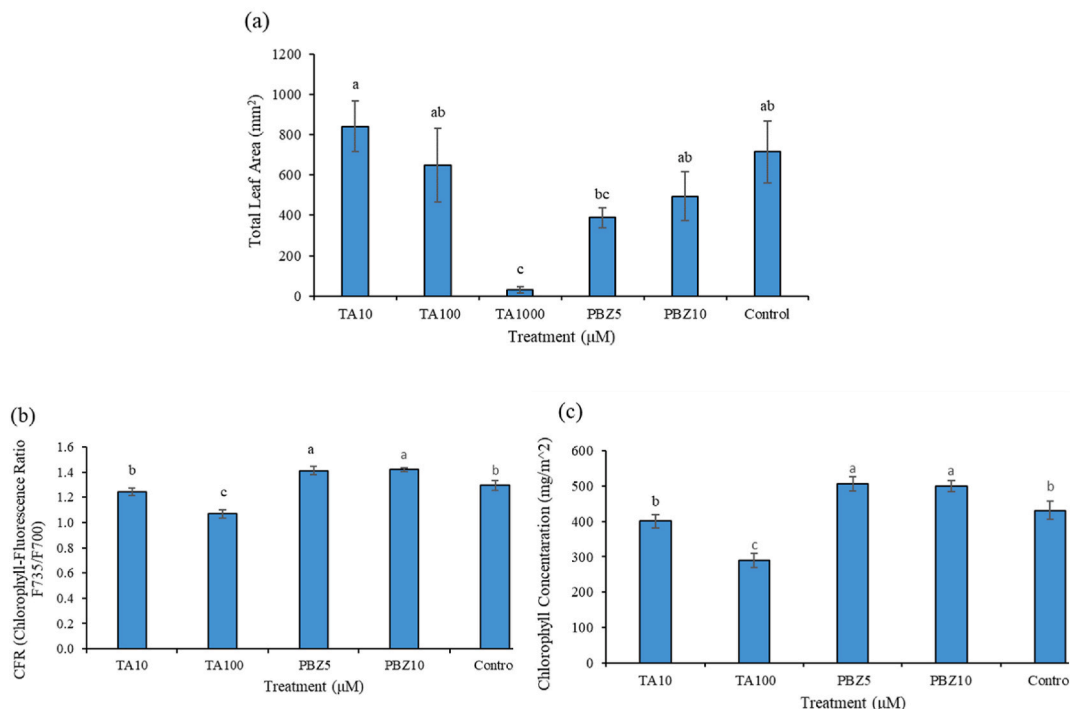
(a)

(b) (c)

Fig. 3. Total Leaf Area (a), CFR (b), and Chlorophyll Concentration (c) Mean comparison (LSD, P ≤ 0.01) of *in vitro Cannabis sativa* L. cv. Roto after application of TA and PBZ treatments. Different letters indicate significant differences between treatments by LSD test. (TA10): Tannic Acid 10 μM, (TA100): Tannic Acid 100 μM, (TA1000): Tannic Acid 1000 μM, (PBZ5): Paclobutrazol 5 μM, (PBZ10): Paclobutrazol 10 μM.

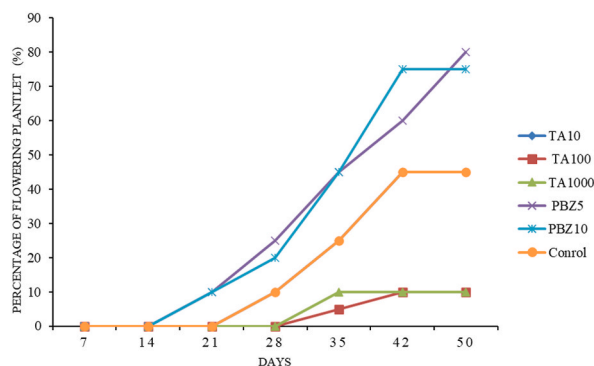**Fig. 4.** Monitor of Flowering Time and percentage of flowering plantlet Based on the Day to flower emergence in *in vitro Cannabis sativa* L. cv. Roto. All plants received a light treatment with a cycle 16:08 (light: dark) from the beginning of the subculture until the start of the 21st day. Afterward, from the 21st day until the end of the harvesting period, they were under a light cycle 12:12 (light: dark). (TA10): Tannic Acid 10 μM, (TA100): Tannic Acid 100 μM, (TA1000): Tannic Acid 1000 μM, (PBZ5): Paclobutrazol 5 μM, (PBZ10): Paclobutrazol 10 μM.

abscisic acid and cytokinins [50]. The positive effect of PBZ application on increasing flowering in citrus has also been confirmed [51]. Furthermore, it is reported that PBZ application significantly increased the number of flower buds and also led to a 22-day earlier flowering in Mango (*Mangifera indica* L.) while reducing the number of vegetative branches [52]. Additionally, it has been observed that using PBZ significantly decreases the length of internode [53,54]. In a study *in vivo* condition, the effect of PBZ on the sunflower plant (*Helianthus annuus* L.) was investigated, which showed the impact of PBZ treatment on the shortening of plants and the increase of the amount of chlorophyll [55].

These results confirm the findings of the previous studies evaluating PBZ in cannabis that demonstrate it significantly promotes floral development [30]. In this experiment, a seven-day long difference was observed between TA and PBZ treatments regarding the appearance of flowers; accordingly, the early flowering in this case can be the result of PBZ application caused by the premature growth of stem and increase in photosynthesis speed [56]. As seen in the results obtained from this experiment, the efficiency of energy transfer in the photosynthetic system (CFR) followed by the concentration of chlorophyll in plants treated via PBZ was at its highest,
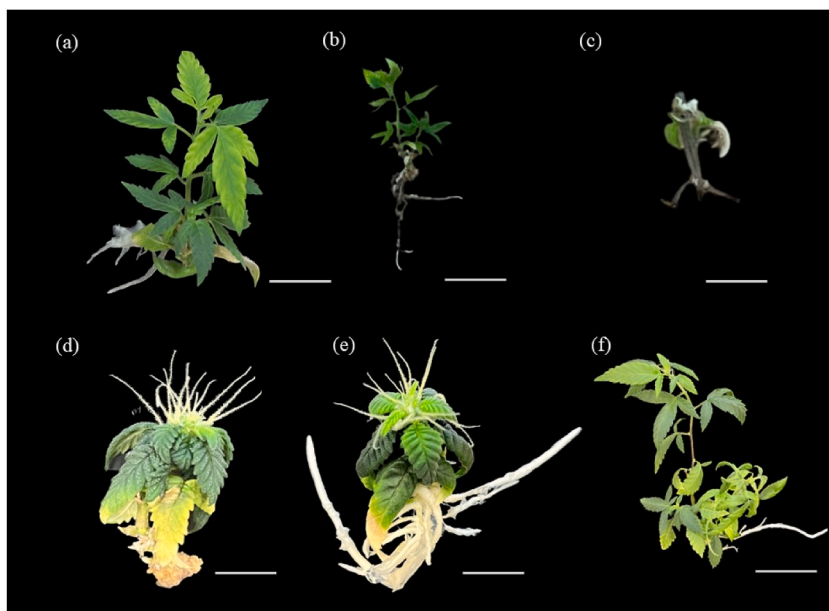
Fig. 5. *In vitro* grown Plantlet of *Cannabis sativa* L. cv. Roto in different treatments at the end of the experiment (day 50) including: (a): Tannic Acid 10 μM, (b): Tannic Acid 100 μM, (c): Tannic Acid 1000 μM, (d): Paclobutrazol 5 μM, (e): Paclobutrazol 10 μM and (f): Control. (bar = 10 mm).
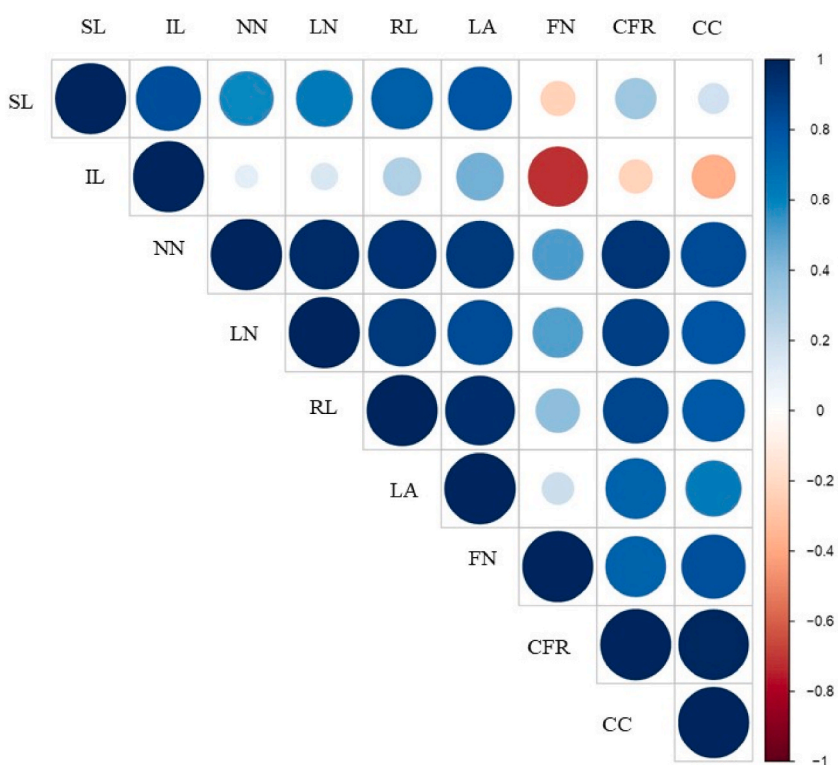

Fig. 6. Heat map Correlation (Pearson) obtained for studied morphological traits of *in vitro* grown plantlet of *Cannabis sativa* L. cv. Roto with Treatments (Tannic Acid and Paclobutrazol). SL: Shoot Length, IL: Internode length, NN: Node Number, LN: Leaf Number, RL: Root Length, LA: Leaf Area, FN: Flowers Number, CFR: Chlorophyll-Fluorescence Ratio F735/F700, CC: Chlorophyll Concentration.

which is in line with the findings of Singh and Singh (2003). Also, the use of PBZ showed the highest amount of chlorophyll in rice plants (*Oryza sativa* L.) [57,58]. Baiton et al. (2024) showed at 14 days, the *in vitro* cannabis seedlings treated with 1 μM and 10 μM PBZ have 70.0 % and 85.0 % of their seedlings in the reproductive phase, respectively. Additionally, they noted a significant difference in the percentage of flowering plantlet between the seedlings treated with PBZ and GA_3, with only 45.8 % of the plants treated with 100 μM PBZ having flower seedlings, while none of the seedlings treated with 100 μM GA_3 had flowers.

TA has been reported to have potential GA_3 inhibitory activity [59] and was evaluated to determine if it could be used as a less toxic alternative to promote flowering in cannabis. However, in this study, TA generally had the opposite effect of PBZ. Further, the responses observed here in response to TA were similar to what was observed in previous work in response to GA_3 [39], although to a lesser extent. Taken together, this suggests that TA is acting as a mild GA_3 agonist rather than an inhibitor. However, it should be noted that TA has previously been reported to have both antagonistic and agonistic properties in respect to GA_3 responses depending on dose [60], so the results of this study are not completely at odds with established literature. Therefore, examinations into the physiological effects of phenolic compounds on plants have demonstrated that their application at low concentrations stimulates growth in plants. When these compounds are used at high concentrations, they act as inhibitors and prevent plant growth [35,61]. This could potentially be explained if TA has weak agonistic interactions with the receptor such that it would act as a competitive inhibitor and reduce the GA_3 response when large amounts of GA_3 are present, while eliciting a weak GA_3 response when low levels of GA_3 are present. In this experiment, no exogenous GA_3 was applied, and TA seemed to elicit a weak GA_3 response. While plants treated with 1000 μM TA displayed reduced plant height, this appeared to be a result of toxicity rather than an inhibitory response based on the dramatic reduction in plant fresh weight, leaf number, and other growth parameters that were not observed in response to PBZ.

5. Conclusion

In this study, we compared the effect of two potential GA_3 inhibitors (TA and PBZ) on the flowering characteristics of cannabis using an *in vitro* system. The main purpose behind this experiment was to evaluate the potential of TA as a natural alternative to PBZ to enhance flowering in cannabis. Like previous results, this study confirmed that PBZ encourages floral development in cannabis, resulting in the production of more flowers, sooner, and in a higher percentage of flowering plantlet, while also resulting in reduced internode length and higher levels of chlorophyll. The highest number of flowers and percentage of flowering plantlet was observed in PBZ which, compared to the control treatment, showed a 59.74 % increase. Despite our initial hypothesis that TA would have similar effects and could serve as a less toxic natural alternative to PBZ, this was not the case. Instead, TA had the opposite effect and appeared to elicit a mild GA_3 response, with longer internodes, reduced chlorophyll content, and reduced floral development and growth. So, increasing TA concentrations up to 100 μM decreased the number of flowers. On the contrary the highest number of leaves (12.5) was found in the presence of TA at a concentration of 100 μM. Despite these findings, it is clear that GA_3 metabolism plays a critical role in the development of flowers in cannabis and serves as a good target for future work, including the development of alternative GA_3 inhibitors to enhance floral development and as a breeding target. For example, optimizing flowering and its timing in cannabis not only aids in achieving fast breeding but also opens up possibilities for floral reversion and even sex reversal. Although the conditions of *in vitro* differ significantly from those of *in vivo*, planning for investigating the effects of these regulators under *in vivo* conditions can be made for the future.

Data availability statement

Data are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Ladan Ajdanian: Writing – review & editing, Writing – original draft, Software, Methodology, Conceptualization. **Hossein Arouiee:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Andrew Maxwell Phineas Jones:** Writing – review & editing, Resources, Project administration, Methodology, Conceptualization. **Mohsen Hesami:** Writing – review & editing, Software, Methodology, Conceptualization. **Hossein Nemati:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Marco Pepe:** Writing – review & editing, Visualization, Software, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36768.

References

[1] I. Kovalchuk, et al., The genomics of Cannabis and its close relatives, Annu. Rev. Plant Biol. 71 (2020) 713–739.
[2] M. Hesami, et al., Advances and perspectives in tissue culture and genetic engineering of cannabis, Int. J. Mol. Sci. 22 (11) (2021) 5671.
[3] A.S. Monthony, et al., The past, present and future of Cannabis sativa tissue culture, Plants 10 (1) (2021) 185.
[4] M. Hesami, et al., Recent advances in cannabis biotechnology, Ind. Crop. Prod. 158 (2020) 113026.
[5] H. Lata, et al., *Thidiazuron-induced high-frequency direct shoot organogenesis of Cannabis sativa L.* In Vitro Cellular &amp, Developmental Biology-Plant 45 (2009) 12–19.
[6] K.F. Piunno, et al., Regeneration of shoots from immature and mature inflorescences of Cannabis sativa, Can. J. Plant Sci. 99 (4) (2019) 556–559.
[7] M. Babaei, L. Ajdanian, B.A. Lajayer, Morphological and phytochemical changes of Cannabis sativa L. affected by light spectra, in: New and Future Developments in Microbial Biotechnology and Bioengineering, Elsevier, 2022, pp. 119–133.
[8] H. Lata, et al., High frequency plant regeneration from leaf derived callus of high Δ9-tetrahydrocannabinol yielding Cannabis sativa L, Planta Med. 76 (14) (2010) 1629–1633.
[9] M.P. Bridgen, W. Van Houtven, T. Eeckhaut, Plant tissue culture techniques for breeding, Ornamental Crops (2018) 127–144.
[10] K. Chadipiralla, et al., Plant tissue culture and crop improvement, Sustainable Agriculture in the Era of Climate Change (2020) 391–412.
[11] I. Sivanesan, B.R. Jeong, Micropropagation and in vitro flowering in Pentanema indicum Ling, Plant Biotechnol. 24 (5) (2007) 527–532.
[12] M. Moher, M. Jones, Y. Zheng, Photoperiodic response of in vitro Cannabis sativa plants, Hortscience 56 (1) (2021) 108–113.
[13] M. Babaei, et al., Characterization of indigenous populations of cannabis in Iran: a morphological and phenological study, BMC Plant Biol. 24 (1) (2024) 151.
[14] N. Gerald, U.K. Frei, T. Lübberstedt, Accelerating plant breeding, Trends Plant Sci. 18 (12) (2013) 667–672.
[15] R. Abdul Fiyaz, et al., Speed breeding: methods and applications. Accelerated Plant Breeding, Cereal Crops 1 (2020) 31–49.
[16] F. Cazzola, et al., Speed breeding in pea (Pisum sativum L.), an efficient and simple system to accelerate breeding programs, Euphytica 216 (11) (2020) 178.
[17] S.H. Mobini, et al., Plant growth regulators improve in vitro flowering and rapid generation advancement in lentil and faba bean, In Vitro Cell Dev. Biol. Plant 51 (2015) 71–79.
[18] K. Samantara, et al., Breeding more crops in less time: a perspective on speed breeding, Biology 11 (2) (2022) 275.
[19] J.A. Wiesinger, et al., An in vivo (Gallus gallus) feeding trial demonstrating the enhanced iron bioavailability properties of the fast cooking manteca yellow bean (Phaseolus vulgaris L.), Nutrients 11 (8) (2019) 1768.
[20] S. Mobini, et al., Shortening the generation cycle in faba bean (Vicia faba) by application of cytokinin and cold stress to assist speed breeding, Plant Breed. 139 (6) (2020) 1181–1189.
[21] J.A. Roberts, Plant Growth Regulators, Springer Science & Business Media, 2012.
[22] C.A. Jaleel, et al., Responses of antioxidant defense system of Catharanthus roseus (L.) G. Don. to paclobutrazol treatment under salinity, Acta Physiol. Plant. 29 (2007) 205–209.
[23] L.-H. Zhu, et al., Changes of leaf water potential and endogenous cytokinins in young apple trees treated with or without paclobutrazol under drought conditions, Sci. Hortic. 99 (2) (2004) 133–141.
[24] M.M. Rady, S.G. Maybelle, Improving barley yield grown under water stress conditions, Research Journal of Recent Sciences ISSN 2277 (2012) 2502.
[25] J. Marshall, et al., The effects of paclobutrazol, abscisic acid, and gibberellin on germination and early growth in silver, red, and hybrid maple, Can. J. For. Res. 30 (4) (2000) 557–565.
[26] R. Gupta, S. Chakrabarty, Gibberellic acid in plant: still a mystery unresolved, Plant Signal. Behav. 8 (9) (2013) e25504.
[27] P. Brian, Effects of gibberellins on plant growth and development, Biol. Rev. 34 (1) (1959) 37–77.
[28] H.M. Ram, Jaiswal, and VS, Induction of male flowers on female plants of Cannabis sativa by gibberellins and its inhibition by abscisic acid, Planta 105 (1972) 263–266.
[29] D. Stone, Cannabis, pesticides and conflicting laws: the dilemma for legalized States and implications for public health, Regul. Toxicol. Pharmacol. 69 (3) (2014) 284–288.
[30] A. Baiton, Novel Strategies for Sustainable Rapid Breeding of Cannabis Sativa L, University of Guelph, 2024.
[31] B. Kaczmarek, Tannic acid with antiviral and antibacterial activity as a promising component of biomaterials—a minireview, Materials 13 (14) (2020) 3224.
[32] N. Aelenei, et al., Tannic acid incorporation in chitosan-based microparticles and in vitro controlled release, J. Mater. Sci. Mater. Med. 20 (2009) 1095–1102.
[33] M.I. Al-Wakeel, Z.R. Murshidy, A.A. Al-Ghanimi, Preparation and characterization of NanohybridCompound from tannic acid and zinc oxide, J. Pharmaceut. Sci. Res. 10 (12) (2018) 3181.
[34] M.R. Corcoran, T. Geissman, B.O. Phinney, Tannins as gibberellin antagonists, Plant Physiol. 49 (3) (1972) 323–330.
[35] C. Tanase, O.-C. Bujor, V.I. Popa, Phenolic natural compounds and their influence on physiological processes in plants, in: Polyphenols in Plants, Elsevier, 2019, pp. 45–58.
[36] M. Hesami, A.M.P. Jones, Modeling and optimizing callus growth and development in Cannabis sativa using random forest and support vector machine in combination with a genetic algorithm, Appl. Microbiol. Biotechnol. 105 (12) (2021) 5201–5212.
[37] A. Friedrichs, et al., SmartFluo: a method and affordable adapter to measure chlorophyll a fluorescence with smartphones, Sensors 17 (4) (2017) 678.
[38] P.J. Mohan, S.D. Gupta, Intelligent image analysis for retrieval of leaf chlorophyll content of rice from digital images of smartphone under natural light, Photosynthetica 57 (2) (2019) 388–398.
[39] F. Vesali, et al., Feasibility of using smart phones to estimate chlorophyll content in corn plants, Photosynthetica 55 (2017) 603–610.
[40] M. Hesami, et al., Modeling and optimizing in vitro seed germination of industrial hemp (Cannabis sativa L.), Ind. Crop. Prod. 170 (2021) 113753.
[41] M.Y. Najafabadi, A. Heidari, I. Rajcan, AllInOne Pre-processing: a comprehensive preprocessing framework in plant field phenotyping, SoftwareX 23 (2023) 101464.
[42] J.A. Teixeira da Silva, D.T. Nhut, Thin cell layers and floral morphogenesis, floral genetics and in vitro flowering, in: Thin Cell Layer Culture System: Regeneration and Transformation Applications, Springer, 2003, pp. 285–342.
[43] I. Kostenyuk, B. Oh, I. So, Induction of early flowering in Cymbidium niveo-marginatum Mak in vitro, Plant Cell Rep. 19 (1999) 1–5.
[44] E. Morales-Martinez, et al., Effect of nitrates alone or with paclobutrazol on flowering induction and production in mango cv. Tommy Atkins, Biotec 22 (2) (2020) 20–27.
[45] C.K. Rai, M. Rana, L. Sharma, Response of plant growth substances and bio-enhancer on the enhancement of bulblet growth under the in-vivo condition of Lilium, Int. J. Phys. Soc. Sci. 35 (14) (2023) 57–68.
[46] A. Erez, Dwarfing peaches by pruning and by paclobutrazol, in: International Workshop on Controlling Vigor in Fruit Trees 146, 1983.
[47] M. Williams, L. Edgerton, Vegetative Growth Control of Apple and Pear Trees with ICI PP333 (Paclobutrazol) a Chemical Analog of Bayleton, 1982.
[48] K.K. Upreti, et al., Hormonal changes in response to paclobutrazol induced early flowering in mango cv. Totapuri, Sci. Hortic. 150 (2013) 414–418.
[49] R. Yadav, et al., Use of paclobutrazol in horticultural crops–a review, Agric. Rev. 26 (2) (2005) 124–132.
[50] G. Murti, K. Upreti, Plant hormones, Advances in plant physiology 3 (2000) 109–148.

[51] A. Martínez-Fuentes, et al., Fruit load restricts the flowering promotion effect of paclobutrazol in alternate bearing Citrus spp, Sci. Hortic. 151 (2013) 122–127.

[52] M.H. Rahman, et al., Applying paclobutrazol and flower bud pruning modify the fruiting time and fruit quality of 'Amrapali'Mango (mangifera indica L.), The Horticulture Journal 92 (3) (2023) 255–260.

[53] W. Wani, et al., Carry-over effect of paclobutrazol on vegetative parameters of sweet cherry, Int. J. Fruit Sci. 11 (4) (2011) 424–429.

[54] G. Steffens, et al., Growth, carbohydrate, and mineral element status of shoot and spur leaves and fruit of 'Spartan'apple trees treated with paclobutrazol, J. Am. Soc. Hortic. Sci. 110 (6) (1985) 850–855.

[55] J. Sarkar, Effect of paclobutrazol on physiology, growth and yield of sunflower (Helianthus annuus L.), Plant Physiology Reports 28 (2) (2023) 231–237.

[56] V. Singh, A. Singh, Effect of paclobutrazol on regularity of bearing in mango (Mangifera indica L.), Physiol. Mol. Biol. Plants 9 (2003) 239–248.

[57] O.A. Alkhateeb, et al., Improving water-deficit stress tolerance in rice (oryza sativa L.) by paclobutrazol exogenous application, Pol. J. Environ. Stud. 33 (3) (2024) 3055–3066.

[58] Y. Zhang, et al., Effects of paclobutrazol seed priming on seedling quality, photosynthesis, and physiological characteristics of fragrant rice, BMC Plant Biol. 24 (1) (2024) 53.

[59] F.B. Green, M.R. Corcoran, Inhibitory action of five tannins on growth induced by several gibberellins, Plant Physiol. 56 (6) (1975) 801–806.

[60] D. Stoms, Effect of polyphenols on shoot and root growth and on seed germination, Biol. Plantarum 24 (1) (1982) 1–6.

[61] A. Kumar, et al., The effect of cadmium tolerant plant growth promoting rhizobacteria on plant growth promotion and phytoremediation: a review, Curr. Microbiol. 80 (5) (2023) 153.