EFFECTS OF DIFFERENT CYTOKININS ON THE MICROPROPAGATION OF *PETUNIA HYBRIDA* L.

ẢNH HƯỞNG CỦA CÁC LOẠI CYTOKININ ĐẾN QUÁ TRÌNH VI NHÂN GIỐNG *PETUNIA HYBRIDA* L.

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Abstract - *Petunia hybrida* L. has beautiful flowers with vibrant and diverse colors. This study evaluated the effects of three cytokinins - kinetin, benzyl adenine (BA), and meta-topolin (mT) - on *in vitro* shoot multiplication of petunia. Nodal explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of cytokinins. After three weeks, all treatments induced shoot formation. The highest shoot proliferation (22 shoots per explant) was obtained with 1.0 mg/L mT, significantly higher than BA (8.25 shoots/explant) and kinetin (6.62 shoots/explant). For rooting, half-strength MS medium without auxin effectively promoted root development. Plantlet survival depended on the cytokinin used during shoot multiplication. Shoots cultured with 1.0 mg/L mT and rooted without auxin had the highest survival rate (84.62%). These findings suggest that mT is an effective cytokinin for micropropagation of petunia, enhancing the shoot multiplication rate and improving both the quality and survival rate of the plantlets.

Key words - Cytokinin*;* meta topolin*; Petunia hybrida* L.; acclimatization

Tóm tắt - *Petunia hybrida* L. (dạ yến thảo) là loài cây có giá trị cảnh quan cao nhờ hoa có màu sắc và hình thái đa dạng. Nghiên cứu nhằm đánh giá ảnh hưởng của ba loại cytokinin (kinetin, benzyl adenine (BA), và meta-topolin (mT)) đến khả năng nhân chồi *in vitro* của dạ yến thảo. Mẫu đốt thân được nuôi cấy trên môi trường MS bổ sung các cytokinin ở các nồng độ khác nhau. Sau 3 tuần, tất cả các nghiệm thức đều hình thành chồi, trong đó môi trường chứa 1,0 mg/L mT cho kết quả vượt trội với trung bình 22 chồi/mẫu, cao hơn đáng kể so với BA (8,25 chồi/mẫu) và kinetin (6,62 chồi/mẫu). Ở giai đoạn ra rễ, môi trường MS ½ không bổ sung auxin giúp chồi ra rễ tốt. Tỉ lệ sống của cây phụ thuộc vào loại cytokinin ở giai đoạn nhân chồi. Chồi nhân nhanh với mT 1,0 mg/L và ra rễ không cần auxin có tỉ lệ sống cao nhất (84,62%). Như vậy, mT mang lại hiệu quả cao trong vi nhân giống dạ yến thảo, giúp tăng hệ số nhân chồi, chất lượng và tỉ lệ sống của cây con.

Từ khóa - Cytokinin; meta topolin; *Petunia hybrida* L.; thích nghi khí hậu

1. Introduction

Petunias (*Petunia hybrida* L.) are commonly grown for ornamental and decorative purposes due to their vibrant flowers, long-lasting blooms, and high commercial value. They are also considered model plant species and are widely used in molecular and genetic research [1]. In addition, petunias serve as subjects for studies on flavonoid biosynthesis and crop improvement [2]. They can be propagated by stem cuttings or by sowing seeds in the soil. However, seed germination rates are relatively low, reaching only about 50–60% [2].

Micropropagation is a crucial and effective technique for producing high-quality plantlets of petunia. This method helps preserve characteristics such as flower colors, longevity, and plant shape [2]. Several *in vitro* propagation studies of this species have been conducted to determine effective explant sterilization methods and culture medium components (including mineral composition, types and concentrations of plant growth regulators, coconut water, and sucrose) [3] - [8]. The Murashige and Skoog (MS) basal medium [9] supplemented with benzyl adenine (BA) (0.1 – 2.0 mg/L) was commonly used during the shoot multiplication stage [5] - [7], while auxin indole-3-butyric acid (IBA) or naphthaleneacetic acid (NAA) was applied during the rooting stage [3], [5]. The study by Tawfik et al. indicated that half-strength MS medium (with reduced macronutrients) was suitable for root induction in petunia shoots [8]. However, some studies reported a low survival rate of acclimatized plants, reaching only 45% under nursery conditions [3].

Among plant growth regulators, cytokinins play a key role in regulating plant development, including organogenesis, particularly shoot formation. Benzyl adenine (BA) is one of the most widely used cytokinins. However, BA has some adverse effects, such as inhibiting root growth and limiting acclimatization in particular plant species. Kinetin, another synthetic cytokinin, has been reported to promote shoot initiation in various plant species, but its use in *Petunia* is limited. Meta-topolin (mT) has emerged as a potential alternative to other cytokinins. This compound can enhance *in vitro* shoot proliferation rates and help overcome challenges in micropropagation. mT delays chlorophyll and protein degradation during senescence [10] and has been shown to outperform BA in species such as *Pistacia vera* L. [11], *Withania somnifera* [12], and *Sesamum indicum* L. [13]. mT has been associated with improved rooting [12], [14], [15], reduced vitrification [11], and enhanced the synthesis of secondary metabolites in *in vitro* cultures [16], [17].

This study aimed to compare the effects of three cytokinins - benzyl adenine (BA), kinetin, and meta-topolin (mT) - on the micropropagation of *Petunia hybrida*, focusing on shoot multiplication, rooting, and acclimatization. The cytokinin concentrations were chosen based on prior studies, typically ranging from 0.5 to 2.0 mg/L, which have been reported to promote shoot proliferation effectively. The objective was to identify the most suitable cytokinin type and concentration for efficient *in vitro* propagation.

2. Materials and Methods

2.1. Materials

Petunia shoots were obtained from the laboratory of the Department of Biotechnology, University of Technology – Vietnam National University, Ho Chi Minh City. The chemicals (BA, kinetin, and meta-topolin) were purchased from Duchefa Biochemie.

The culture room was maintained at 25 ± 2°C with a light intensity of 3000 lux under a 12-hour photoperiod.

2.2. Methods

2.2.1. Shoot multiplication

Nodal explants excised from 4-week-old petunia shoots, which had been subcultured three times on MS basal medium, were cultured on MS medium supplemented with BA, kinetin, or mT at concentrations of 0.5, 1.0, 1.5, and 2.0 mg/L. The control group was cultured on MS medium without plant growth regulators. Shoot number and shoot length were evaluated after a 3-week culture period.

2.2.2. Root formation

Three-week-old shoots, approximately 4 cm in height, from treatments showing effective shoot proliferation were selected for rooting. The culture medium consisted of half-strength MS (with macronutrients reduced by half) supplemented with IBA at 0, 0.5, and 1.0 mg/L. After four weeks of culture, plant height, number of roots, and root length were recorded.

2.2.3. Acclimatization

The plantlets used for acclimatization were derived from the rooting experiment and had 3 to 5 leaves. The growing substrate consisted of a 1:1 mixture of cow manure and coconut coir. Before transplanting, the plantlets were thoroughly rinsed to remove any residual agar from the culture medium. The cultivation conditions were as follows: light intensity was reduced by 80% using shading, with daytime temperatures maintained at 33 ± 2°C and nighttime temperatures at 25 ± 2°C. These temperature conditions were not artificially controlled but reflected the natural ambient climate at the time of the experiment. During the first week, plants were irrigated twice daily; thereafter, irrigation was reduced to once per day. After four weeks of transplantation, parameters such as survival rate, number of roots, root length, and plant dry weight were recorded and analyzed.

2.2.4. Statistical analysis

The experiment was conducted using a completely randomized design (CRD) with 10 plantlets per treatment and three replicates per treatment, resulting in a total of 30 plantlets per treatment. For each treatment, data were collected from all 30 plantlets (n = 30) for each parameter measured. Data were statistically analyzed using SPSS software, and means were compared using Duncan's multiple range test (DMRT) at a significance level of $p \leq 0.05$. The results presented in this study are expressed as mean values ± standard deviation (SD).

3. Results and Discussion

3.1. **Effects of cytokinins on shoot multiplication**

The results obtained after three weeks of culture are presented in Table 1. Statistical analysis (ANOVA followed by Duncan's test) confirmed that shoot number and length differed significantly ($p < 0.05$) among the cytokinin treatments. The control medium (without cytokinin) produced only a few elongated shoots with minimal branching and long leaves. In contrast, media supplemented with cytokinins significantly enhanced shoot proliferation. For all three cytokinin types, the number of shoots per explant increased as cytokinin concentrations increased up to 1.0 mg/L. However, concentrations above 1.0 mg/L resulted in a decline in shoot number (Table 1), accompanied by reduced shoot length and smaller leaves (Figure 1). Thus, excessively high cytokinin concentrations inhibited shoot proliferation. Nguyen also observed that 1 mg/L BA combined with 0.2 mg/L IBA resulted in a high shoot multiplication rate in petunia [6].

Table 1. *Effect of cytokinins on shoot multiplication*

Cytokinin (mg/L)		Shoot number/explant	Shoot length (cm)
Control		2.00 ± 00^{g}	5.41 ± 0.13^{a}
Kinetin	0.5	3.50 ± 1.69^{fg}	3.21 ± 0.40^{bcd}
	1.0	6.62 ± 1.92^{cde}	3.66 ± 0.74^{b}
	1.5	4.38 ± 1.06^{degf}	3.65 ± 0.22^{b}
	2.0	3.62 ± 1.19^{fg}	3.39 ± 0.94^{bc}
BA	0.5	6.25 ± 1.28^{cde}	3.13 ± 0.32^{bcd}
	1.0	8.25 ± 1.98^{c}	3.06 ± 0.94^{cd}
	1.5	4.79 ± 1.00^{ef}	2.80 ± 0.35^{de}
	2.0	4.25 ± 1.04^{efg}	2.40 ± 0.25^{e}
mT	0.5	6.88 ± 2.14^{cd}	2.80 ± 0.47^{de}
	1.0	22.00 ± 3.25^{a}	2.43 ± 0.21^{e}
	1.5	10.63 ± 5.24^{b}	2.70 ± 0.49^{de}
	2.0	10.87 ± 2.53^{b}	1.81 ± 0.40^{e}

Different letters within the same column indicate significant differences at $p < 0.05$

Among the tested cytokinins, shoot number tended to be highest on media supplemented with mT, followed by BA, and lowest with kinetin. The highest shoot number was observed on the medium supplemented with 1.0 mg/L mT, with an average of 22 shoots per explant, which was 11 times higher than the control. These shoots were numerous and exhibited small green leaves (Figure 1-k). In comparison, the maximum shoot numbers recorded at 1.0 mg/L BA and kinetin were 8.25 and 6.62 shoots per explant, respectively. The superior shoot multiplication ability of BA compared to kinetin was reported by Kamal [18]. Different cytokinins exhibit varying binding affinities to histidine kinases, their specific receptors in plants. Consequently, the signal transduction intensity at the same concentration can differ, leading to variable shoot induction effects among cytokinins [19].

Shoot elongation was most significant in the control treatment (5.41 cm). Overall, shoots cultured on kinetin-containing media exhibited greater elongation than those treated with BA, while the shortest shoots were recorded in mT-supplemented treatments. Several other studies have also shown that kinetin tends to promote greater shoot elongation compared to BA, as observed in *Chrysanthemum × grandiflorum* Ramat. Kitam [20], *Cucumis sativus* [21].

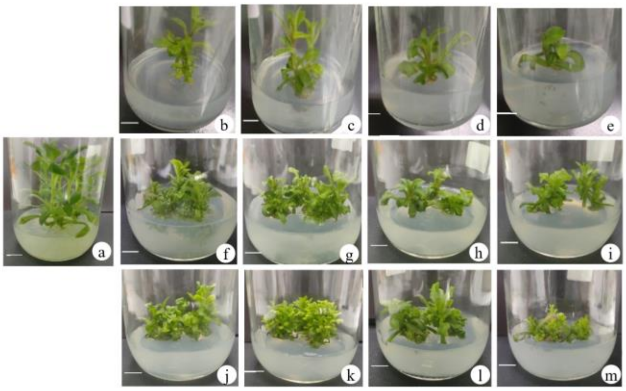

Figure 1. *Shoot proliferation after 3 weeks of culture (scale bar 1 cm)*

a. control; b-e: 0.5, 1.0, 1.5, 2.0 mg/L kinetin; f-i: 0.5, 1.0, 1.5, 2.0 mg/L BA; j-m:0.5, 1.0, 1.5, 2.0 mg/L mT

mT has rarely been used in the micropropagation of *Petunia.* mT has been recognized as a highly effective cytokinin for shoot induction across various medicinal, ornamental, and aromatic plant species. Its efficacy compared to other cytokinins is primarily attributed to its distinct molecular structure. Unlike BA, mT possesses an additional hydroxyl group at the meta position of the benzyl ring, enabling the formation of O-glucoside metabolites, non-toxic storage forms of cytokinins [10]. The conversion of O-glucosides enables mT to prevent localized accumulation and remain in a readily convertible form, facilitating its activation when required. This mechanism contributes to an increased shoot proliferation rate in plant tissue culture. The results of our study are consistent with previous research on other plant species, indicating the high effectiveness of mT in micropropagation protocols. In the micropropagation of *Pistacia vera* L., mT resulted in a shoot multiplication rate six times higher than that achieved with BA. Plantlets cultured with mT showed higher levels of biologically active endogenous cytokinins compared to those treated with BA [11]. Furthermore, mT has also been reported to enhance shoot proliferation and to stimulate chlorophyll and protein biosynthesis in *Withania somnifera* [12].

Among the tested cytokinins, 1.0 mg/L mT produced the most effective results. In addition, media supplemented with 1.0 mg/L BA and 1.0 mg/L kinetin also promoted high shoot numbers. Therefore, shoots obtained from these three treatments were selected for subsequent experiments.

3.2. Root formation

Shoots derived from treatments containing 1.0 mg/L kinetin, BA, or mT were assessed for their rooting ability on media supplemented with IBA at concentrations ranging from 0 to 1.0 mg/L. After four weeks of culture, all treatments demonstrated a 100% rooting success rate. Plantlets cultured on auxin-free media exhibited greater height and developed healthy root systems (Figure 2). However, the supplementation of IBA at 0.5 or 1.0 mg/L reduced plant height and root length compared to explants cultured on an auxin-free medium (Table 2).

***Table 2.** Effects of auxin on rooting*

Shoots	IBA (mg/L)	Plant height (cm)	Root number/ shoot	Root length (cm)
Kinetin derived	0	7.16 ± 1.04^{ab}	17.75 ± 5.87^{b}	6.48 ± 0.93^{a}
	0.5	6.09 ± 1.16^{c}	26.88 ± 5.44^{a}	1.80 ± 0.43^{c}
	1.0	6.31 ± 1.11^{bc}	11.25 ± 3.49^{cd}	1.34 ± 0.39^{c}
BA derived	0	7.59 ± 1.01^{a}	20.13 ± 5.49^{b}	5.66 ± 2.30^{ab}
	0.5	3.95 ± 0.86^{d}	20.00 ± 3.71^{b}	1.95 ± 0.24^{c}
	1.0	4.47 ± 0.66^{d}	8.63 ± 3.93^{d}	0.99 ± 0.18^{c}
mT derived	0	7.69 ± 0.80^{a}	20.00 ± 3.93^{b}	5.31 ± 0.70^{b}
	0.5	5.63 ± 0.23^{c}	29.38 ± 6.41^{a}	1.79 ± 0.37^{c}
	1.0	5.50 ± 1.41^{c}	15.00 ± 4.66^{bc}	1.14 ± 0.34^{c}

Different letters within the same column indicate significant differences at $p < 0.05$

The highest number of roots was observed in the growth medium supplemented with 0.5 mg/L IBA (ranging from approximately 20 to 29 roots). This was followed by the auxin-free treatment (17–20 roots), and the lowest root numbers were recorded in the 1.0 mg/L IBA treatment (averaging 8–15 roots). Among these, explants derived from media containing 1.0 mg/L mT or 1.0 mg/L kinetin and rooted with 0.5 mg/L IBA showed the highest root numbers, with 29.38 and 26.88 roots per explant, respectively.

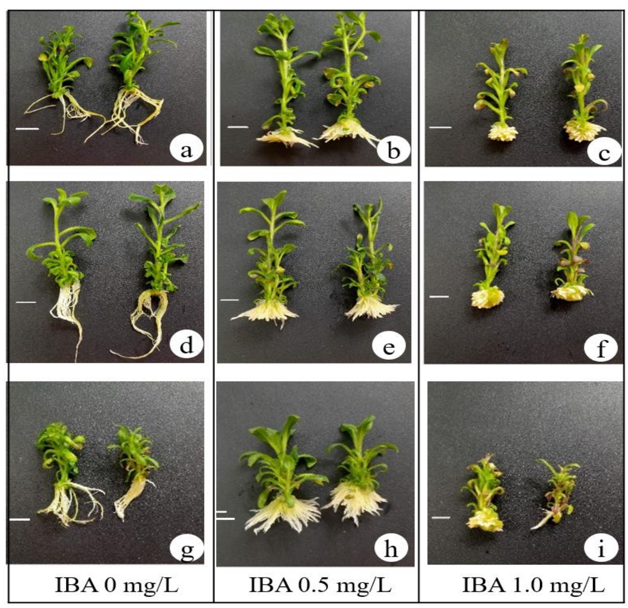

Figure 2. *Root formation after 4 weeks of culture (scale bar 1 cm)*

a, b, c: kinetin-derived shoots; d, e, f: BA-derived shoots; g, h, i: mT-derived shoots

In the absence of IBA, plantlets showed the most significant height, dark green leaves, and well-developed roots (Figure 2 – a, d, g). On medium containing 0.5 mg/L IBA (Figure 2 – b, e, h), plantlets were shorter, with more numerous and thicker roots, although root length was reduced. At 1.0 mg/L IBA (Figure 2 – c, f, i), plantlets had the smallest size; leaves were small and tended to become yellowish or pale, and root development was poor, with fewer and shorter roots. These results suggest that high auxin concentrations inhibited the development of shoots, leaves, and roots. While a low level of auxin promotes root elongation, excessive auxin may exert an inhibitory effect on root formation [22]. In contrast to our findings, Farooq reported that 1.0 mg/L IBA was optimal for inducing root formation in *Petunia* shoots under *in vitro* conditions [5]. In our study, endogenous auxin levels may have been sufficient for root formation; therefore, the supplementation of 1 mg/L IBA had a detrimental effect on rooting. Additionally, several other studies have also reported that petunia shoots require low auxin concentrations for effective rooting. For instance, *Petunia × hybrida* F1 'Opera Supreme Pink Morn' formed roots most effectively with 0.1 mg/L NAA supplementation [7]. Moreover, Borovaya reported that petunia shoots rooted most effectively on hormone-free medium [2].

The present study revealed that shoots regenerated on mT- and kinetin-supplemented media showed improved rooting at 0.5 mg/L IBA compared to those originating from BA-based media. Consistent with this finding, Panley reported that in *Quercus leucotrichophora* L., kinetin-derived shoots rooted more effectively than those developed on BA-containing media [23]. High concentrations of BA during the shoot multiplication phase can lead to cytokinin residue in the tissues, resulting in hormonal imbalance when transferred to the rooting medium [24]. On the other hand, mT has been shown to enhance both the quality and quantity of regenerated shoots, promote higher levels of photosynthetic pigments, and improve biomass accumulation relative to plantlets produced on BA-enriched media. This may be attributed to mT's ability to slow down leaf senescence and increase chlorophyll content, thereby supporting better shoot and root development [13], [25]. mT has been reported to enhance both shoot multiplication and rooting, showing greater effectiveness than BA in *Salvia sclarea* [14], *Pterocarpus marsupium* [25].

In this experiment, plantlets cultured on media without IBA or supplemented with 0.5 mg/L IBA exhibited greater height and healthy root systems. These plantlets were selected for transfer to the nursery for acclimatization.

3.3. Acclimatization

Table 3. *Ex vitro growth of plantlets rooted on IBA-free medium after four weeks*

Treatment	Survival (%)	Root number	Root length (cm)	Dry weight (g)
kinetin derived	54.17	39.00 ± 4.27^a	5.18 ± 0.26^a	0.10 ± 0.03^a
BA derived	28.57	34.00 ± 6.56^a	5.43 ± 0.49^a	0.14 ± 0.03^a
mT derived	84.62	36.33 ± 6.03^a	5.43 ± 0.59^a	0.16 ± 0.03^a

Different letters within the same column indicate significant differences at $p < 0.05$

When transplanted to the nursery, plantlets rooted with 0.5 mg/L IBA showed poor development. The survival rate of plantlets derived from 1.0 mg/L mT and 1.0 mg/L kinetin was 25%, while no survival was recorded for plantlets originating from 1.0 mg/L BA. This may be due to the underdeveloped root systems observed in these treatments, which included short roots lacking lateral roots and root hairs (Figure 2 – b, e, h). As a result, the plantlets were likely unable to absorb sufficient water and nutrients under nursery conditions.

Meanwhile, plantlets that formed roots in treatments without auxin showed better vitality. The recorded parameters are presented in Table 3.

The results indicate that the type of cytokinin used during the shoot multiplication stage significantly impacted the survival rate of plantlets. The highest survival rate was observed in plantlets derived from 1.0 mg/L mT (84.62%), while those cultured with 1.0 mg/L BA showed the lowest survival (28.57%). Thus, mT contributed to improved plantlet vigor. Statistical analysis revealed no significant differences among treatments in terms of root number, root length, or dry weight. In treatments where rooting occurred on IBA-free medium, plantlets exhibited strong stems, vibrant green leaves, and long, abundant roots. Among them, plantlets obtained from 1.0 mg/L mT displayed notable morphology, with well-balanced shoot and root development. These plantlets had thicker stems and larger leaves compared to those originating from BA or kinetin (Figure 3).

Figure 3. *Plantlets after 3 weeks of growth in the nursery (scale bar = 1 cm)*

a. kinetin-derived plantlets, b. BA-derived plantlets, c. mT-derived plantlets

The type and concentration of cytokinin play a significant role in the acclimatization of plantlets. Among the commonly used cytokinins, BA is favored due to its low cost and widespread availability. However, this compound has been reported to inhibit root formation, leading to poor acclimatization rates in many plant species [25]. Shoots developed on media containing synthetic cytokinins such as BA tend to accumulate potentially toxic metabolites at the basal region (where rooting occurs), such as 6-benzylamino-9-β-D-glucopyranosylpurine, which may hinder root development and reduce the acclimatization success of plantlets [13], [26].

In contrast, plantlets derived from media supplemented with mT exhibited enhanced growth performance, possibly due in part to the positive effects of this compound on photosynthetic processes. A well-developed photosynthetic system likely contributes to improved acclimatization of plantlets. mT has been shown to enhance shoot quality and plantlet survival during acclimatization by promoting chloroplast differentiation and reducing chlorophyll degradation [10]. Additionally, mT may inhibit cytokinin dehydrogenase enzymes, enhance cell division activity, minimize shoot abnormalities, increase photosynthetic pigment content and leaf biomass, and delay senescence. These effects help plantlets accumulate essential compounds needed to support autotrophic growth under both *ex vitro* and *in vivo* conditions [26]. In the micropropagation of *Withania somnifera*, plantlets derived from media containing mT had higher chlorophyll content compared to those from BA-treated media [12]. Jayaprakash et al. conducted anatomical observations of leaves from shoots multiplied with BA and mT. Leaves from BA treatments exhibited more abnormalities, including underdeveloped stomata, reduced vein and trichome density, a poorly developed cuticle, and poorly differentiated ground and vascular tissues. In contrast, *in vitro* leaves from mT-containing media showed normal stomata, a thicker cuticle, and well-developed vascular and ground tissues. Plantlets derived from media supplemented with mT also showed a 100% survival rate during acclimatization [26].

4. Conclusion

In the *in vitro* propagation of petunia, the type of cytokinin influenced not only shoot proliferation but also rooting ability and plantlet vigor during the acclimatization stage. Among the tested cytokinins, 1.0 mg/L meta-topolin (mT) was the most effective for shoot multiplication. Shoot proliferation using BA and kinetin also showed the best results at a concentration of 1.0 mg/L, with BA being more effective than kinetin after three weeks of culture. Shoots developed well-formed roots when cultured either on hormone-free medium or in the presence of 0.5 mg/L IBA. Notably, explants previously cultured on media supplemented with 1.0 mg/L mT or 1.0 mg/L kinetin and subsequently rooted on 0.5 mg/L IBA produced the highest number of roots. When transferred to the nursery, plantlets from the 1.0 mg/L mT treatment showed the highest survival rate, followed by those from kinetin, while plantlets originating from the BA treatment showed the lowest survival rate.

These findings suggest that mT can be a suitable alternative to conventional cytokinins in the micropropagation of petunia.

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