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EVALUATION OF DIFFERENT TYPES OF CYTOKININS AS ZEATIN REPLACEMENT ON *IN VITRO* PROLIFERATION OF *VACCINIUM DUNALIANUM*

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Abstract

In plant tissue culture, cytokinin is an important limiting factor for rapid proliferation, but the effects of different types of phytokinins on the proliferation of different plants vary. Isoprenoid cytokinin is more valid for shoot initiation in *Vaccinium* species, the purpose of our research was to study whether *Vaccinium* dunalianum has a preference for Zeatin. *V. dunalianum,* which belongs to Ericaceae, is rich in caffeoylarbutin derivatives. In the early stage, the proliferation system of *V. dunalianum* was established using zeatin (ZT). In this study, *V. dunalianum* was used as experimental material to evaluate the effects of substituting kinetin (Kin), 6-benzylaminopurine (BA), or thidiazuron (TDZ) for ZT in the shoot proliferation stage of *V. dunalianum*. The proliferation rate of *V. dunalianum* decreased when ZT was completely replaced by the other types of cytokinins. When ZT was partially replaced by Kin, 2.0 mg·L^{-1} Kin and 0.5 mg·L^{-1} ZT was discovered to be the optimal combination, and the effective multiplication rate was 3.40. When ZT was partially replaced by BA, 1.0 mg·L^{-1} BA and 0.5 mg·L^{-1} ZT was the best combination, and the effective multiplication rate was 2.26. When ZT was partially replaced by TDZ, malformed buds appeared. Of the combinations tested, 1.0 mg·L^{-1} Kin, 1.0 mg·L^{-1} BA, and 0.1 mg·L^{-1} ZT led to the greatest shoot proliferation rate (4.32), and the growth was normal. In conclusion, ZT is preferred for shoot multiplication of *V. dunalianum*. When ZT was added to the medium, the propagation of *V. dunalianum* was easier and the growth of seedlings was better. Results could provide technical support for reducing the production cost of tissue culture seedlings of *V. dunalianum* and a certain reference for selection of tissue culture formulas of other *Vaccinium* plants.

Key words: *Vaccinium dunalianum*; Cytokinins; Proliferation; Replacement.

Introduction

Plant hormones are chemical substances that are vital for controlling plant growth and development. In general, plant hormones can be divided into six categories, including auxins, gibberellic acid, phytokinins, brassinosteroids (BR), ethene, and ABA (Kucera *et al*., 2005). Among phytohormones, auxin and cytokinin are considered the most vital for controlling growth and development in plant tissue culture; these substances can regulate the growth rate of individual parts and integrate them into the form we need (Davies, 1995; North *et al*., 2012). The rate of phytokinins to auxin in nutritional medium affects the morphogenetic process in plant tissue cultivation (Seldimirova *et al*., 2016). For many plants, high auxin to phytokinin ratios tend to promote early rooting and favor shoot formation (Aina *et al*., 2015). However, the effects of auxin and cytokinin are seldom specific in the eventual impact on growth and development, and the reactions of cells, tissues, and organs *In vitro* vary depending on cultivation status, type of exophyte, and genetype (Gaspar *et al*., 1996). According to culture stage and plant species, selecting the best type and quantity of auxin or cytokinin is crucial for establishment of a rapid propagation system.

An efficient and reliable method for shoot proliferation is the primary objective of micropropagation after *In vitro* cultivation establishment. For the proliferation of woody plants, optimization of the proliferation formula takes a long time. The regeneration of many plants is noted to be genetype specificity due to the changes among cultivars' reaction to *In vitro* status (Manrique *et al*., 2013; Masekesa *et al*., 2016). In addition, different hormone types and combinations can be used for the same plant proliferation formula, indicating the strong versatility of hormone types. However, an interesting phenomenon is discovered in the plant tissue cultivation of *Vaccinium* species; that is, the optimal proliferation formulas reported were ZT or 2-isopentenyladenine (2iP), which was the most frequent cytokinin type (Gonzalez *et al*., 2000; Debnath, 2004, 2007). In this regard, an appropriate *In vitro* regeneration method of *Vaccinium dunalianum* by using ZT was developed in our previous study (Luo *et al*., 2014). Whether a plant has a preference for a certain type of hormone remains unknown.

ZT is more valid for shoot initiation in *Vaccinium* species (Reed & Abdelnour-Esquivel, 1991; Gonzalez *et al*., 2000; Debnath, 2004; Debnath & Mcrae, 2005), however, it is a very expensive cytokinin compared. The purpose of the present research was to explore the replacement potency of other types of cytokinins on ZT in the shoot proliferation stage of *V. dunalianum*. Cytokinin treatments included Kin with weak division ability, BA commonly used in engineering production, and TDZ with strong division ability. *V. dunalianum* was used to evaluate the replacement effect of the three cytokinins on ZT. Results can provide scientific guidance for screening of micropropagation formula for *Vaccinium* species and reducing the production cost to a certain extent.

Materials and Methods

Plant material: *In vitro* seedlings of *V. dunalianum* cultured in Key Laboratory of National Forestry and Grassland Administration on Biodiversity Conservation in

Southwest China (Latitude 25°3′55.16" N and Longitude 102°45′20.12" E) were used as the experimental material (Fig. 1A). Seedlings with basically the same growth were cultured on woody plant medium (2.78 g·L^{-1}, Production of Hangzhou Anhe Technology Co., Ltd. in 2019) containing 1.5 mg·L^{-1} ZT to reduce the experimental error. After 60 d of cultivation on the medium, a sufficient number of shoots (4.0-4.5 cm long) were obtained for subsequent experiments.

Effect of replacing ZT by different concentrations of Kin: Shoots were cultivated on a multiplication medium, namely, WPM added with various thickness of Kin (2.0, 3.0, 4.0 mg·L^{-1}) combining ZT (0.0, 0.1, 0.5 mg·L^{-1}). Nine treatments were employed, where 15 cultivation flasks, 5 shoots per flask, were utilized in each treatment. The entire treatments were finished in 3 duplications. Posterior to 60 d of cultivation, the quantity of shoots and shoot height were documented.

Effect of replacing ZT by different concentrations of BA: Shoots were multiplied on WPM containing diverse BA thickness (1.0, 2.0, 3.0 mg·L^{-1}) and ZT (0.0, 0.1, 0.5 mg·L^{-1}) to evaluate the replacement effect of BA on ZT. Nine treatments were employed, where 15 cultivation flasks, 5 shoots per flask, were utilized in each treatment. The entire treatments were finished in 3 duplications. Posterior to 60 d of cultivation on the medium, the quantity of shoots and shoot height were documented.

Effect of replacing ZT by different concentrations of TDZ: Shoots with basically the same growth status were cultured on WPM added with various TDZ thickness (0.5, 1.0, 1.5 mg·L^{-1}) combining ZT (0, 0.1, 0.5 mg·L^{-1}) to evaluate the replacement effect of TDZ on ZT. Nine treatments were employed, where 15 cultivation flasks, 5 shoots per flask, were utilized in each treatment. The entire treatments were finished in 3 duplications. Posterior to 60 d of cultivation on the medium, the quantity of shoots and shoot height were documented.

Effect of full or partial replacing ZT by various sets of cytokinins: Shoots were placed onto WPM added with various Kin thickness (0.0, 1.0, 1.5 mg·L^{-1}), BA (0.0, 0.5, 1.0 mg·L^{-1}), and TDZ (0.0, 0.3, 0.8 mg·L^{-1}) combining ZT (0.0, 0.1 mg·L^{-1}). Sixteen treatments were employed, where 15 cultivation flasks, 5 shoots per flask, were utilized in each treatment. The entire treatments were finished in 3 duplications. Posterior to 60 d of cultivation on the medium, the quantity of shoots and shoot height were documented. The proliferative ratio was computed below:

$$\text{Proliferation rate} = \frac{\text{Number of shoots in subculture}}{\text{Number of inoculations}} \times 100.$$

Culture conditions: The entire media herein comprised 30 g·L^{-1} saccharose and 5 g·L^{-1} agar. The pH of the intermediary was modified to 5.2 by adding HCL or NaOH prior to autoclaving at 121°C for twenty minutes. The entire cultivation process was kept under 25 ± 2°C at an ambience light of 1500-2000 lx light by cool white fluorescence lights and a light rhythm of 14 h light/10 h darkness. ZT was supplemented via filtration posterior to the autoclaving of the intermediary.

Fig. 1. Seedlings cultivated on WPM intermediary with diverse types of cytokinins.
A: Seedlings cultured on WPM medium containing 1.5 mg·L^{-1} ZT; **B**: Shoot multiplication on WPM intermediary added 4.0 mg·L^{-1} Kin; **C**: Shoot multiplication on WPM intermediary added 2.0 mg·L^{-1} Kin combining 0.5 mg·L^{-1} ZT; **D**: Shoot multiplication on WPM intermediary with 3.0 mg·L^{-1} BA; **E**: Shoot proliferation on WPM medium with 1.0 mg·L^{-1} BA and 0.5 mg·L^{-1} ZT; **F**: Shoot proliferation on WPM medium with 0.5 mg·L^{-1} TDZ; **G**: Shoot proliferation on WPM intermediary with 0.5 mg·L^{-1} TDZ combining 0.5 mg·L^{-1} ZT; **H**: Shoot multiplication on WPM intermediary 1.0 mg·L^{-1} Kin, 1.0 mg·L^{-1} BA combining 0.1 mg·L^{-1} ZT.

Table 1. Effect of full or partial replacing ZT by Kin.

Treatment	Kin(mg·L^{-1})	ZT (mg·L^{-1})	Proliferation rate	Average seedling height (cm)	Degree of the callus growth	Degree of seedling
1	2.0	0.0	1.42 ±0.19d	4.0	-	**
2	3.0	0.0	1.58 ±0.36d	3.7	-	**
3	4.0	0.0	1.38 ±0.26d	3.3	-	**
4	2.0	0.1	2.20 ±0.36cd	3.3	+	***
5	3.0	0.1	3.16 ±0.74bc	4.5	-	***
6	4.0	0.1	2.26 ±0.39cd	5.2	+	***
7	2.0	0.5	3.40±1.00b	6.9	+	***
8	3.0	0.5	2.50±0.65bcd	5.8	+	***
9	4.0	0.5	3.24±0.94bc	4.3	+	***
CK	0.0	1.5	4.46 ±0.40a	4.4	+	***

Notes: means with the identical letter in the identical column aren't diverse in terms of statistics (p<0.05).The numerical results denote average ±SD. (-) indicates no callus, (+) indicates some callus and (*) indicates status of seedling growth. *: poor, **: good, ***: excellent

Table 2. Effect of full or partial replacing ZT by BA.

Treatment	BA (mg·L^{-1})	ZT (mg·L^{-1})	Proliferation rate	Average seedling height (cm)	Degree of the callus growth	Degree of seedling
1	1.0	0.0	1.38 ± 0.25c	3.1	-	*
2	2.0	0.0	1.42 ± 0.19c	3.2	-	*
3	3.0	0.0	1.40 ± 0.16c	2.5	-	*
4	1.0	0.1	1.66 ± 0.23bc	3.9	+	**
5	2.0	0.1	2.06 ± 0.36bc	2.9	-	***
6	3.0	0.1	2.24 ± 0.27b	2.9	+	***
7	1.0	0.5	2.26 ± 0.84b	3.5	+	***
8	2.0	0.5	2.08 ± 0.40bc	3.7	+	***
9	3.0	0.5	1.76 ± 0.60bc	2.7	-	*
CK	0.0	1.5	4.46 ± 0.40a	4.4	+	***

Notes: means with the identical letter in the identical column aren't diverse in terms of statistics (p<0.05).The numerical results denote average ±SD. (-) indicates no callus, (+) indicates some callus and (*) indicates status of seedling growth. *: poor, **: good, ***: excellent.

Statistics

The entire data were assayed via Excel 2010 and assessed via ANOVA on SPSS 20.0 at 5% probabilistic level.

Results

Replacement results of Kin on ZT: The shoots were cultivated on shoot WPM. The utilization of Kin as phytokinin for *In vitro* shoot proliferative activity was explored in *V. dunalianum* to reduce and replace ZT (Table 1). When ZT was absolutely replaced by Kin, ANOVA revealed that the increase in the Kin concentration had no remarkable (p<0.05) potency on the proliferative rate, which was 1.38 when 4.0 mg·L^{-1} Kin was used (Fig. 1B). The addition of ZT in WPM containing Kin was discovered to be valid for shoot proliferative activity. With increasing Kin concentration, the proliferation rate also increased. When 2.0 mg·L^{-1} Kin combining 0.5 mg·L^{-1} ZT was applied, the proliferation rate reached 3.40 and the highest mean seedling height (6.9 cm) was acquired (Fig. 1C). However, callus was present at the bottom of the shoots cultured on WPM intermediary added with 2.0 mg·L^{-1} Kin combining 0.5 mg·L^{-1} ZT.

Replacement results of BA on ZT: The efficacy of BA in the shoot multiplication stage of *V. dunalianum* was investigated as an alternative to ZT. When ZT was absolutely replaced by BA, ANOVA revealed that the increase in the BA concentration had no remarkable (p<0.05) effect on the proliferative rate (Table 2). If the BA thickness registered 3.0 mg·L^{-1}, the proliferative ratio of the shoots would be only 1.40 (Fig. 1D). The addition of ZT to WPM medium containing BA was discovered to be effective for shoot proliferative activity. WPM added with 1.0 mg·L^{-1} BA in combination with 0.5 mg·L^{-1} ZT was found to be the optimal intermediary for shoot proliferation (Fig. 1E) and growth (2.26 and 3.5 cm, respectively). No significant difference in proliferation rate was found compared with that in intermediary added with 3.0 mg·L^{-1} BA and 0.1 mg·L^{-1} ZT (2.24).

Replacement results of TDZ on ZT: The shoots cultivated on WPM intermediary with diverse thickness of TDZ and ZT were assessed after 60 d of culture (Table 3). The addition of TDZ was not conducive to shoot proliferation. The lowest proliferation rate (1.3-1.5) was obtained using different concentrations TDZ in WPM medium (Fig. 1F-G), which was significantly lower than those produced with the control (4.46). The addition of TDZ also resulted in the appearance of malformed buds.

Table 3. Effect of full or partial replacing ZT by TDZ.

Treatment	TDZ ($mg \cdot L^{-1}$)	ZT($mg \cdot L^{-1}$)	Proliferation rate	Average seedling height (cm)	Degree of the callus growth	Degree of seedling
1	0.5	0.0	1.50 ± 0.42b	2.8	-	*
2	1.0	0.0	1.40 ± 0.31b	2.7	-	*
3	1.5	0.0	1.30 ± 0.17b	2.0	-	*
4	0.5	0.1	1.34 ± 0.11b	2.2	-	*
5	1.0	0.1	1.38 ± 0.25b	2.0	-	*
6	1.5	0.1	1.34 ± 0.11b	1.8	-	*
7	0.5	0.5	1.32 ± 0.13b	4.0	-	*
8	1.0	0.5	1.42 ± 0.22b	3.9	-	*
9	1.5	0.5	1.30 ± 0.17b	2.5	-	*
CK	0.0	1.5	4.46 ± 0.40a	4.4	-	***

Notes: means with the identical letter in the identical column aren't diverse in terms of statistics (p<0.05).The numerical results denote average ±SD. (-) indicates no callus, (+) indicates some callus and (*) indicates status of seedling growth. *: poor, **: good, ***: excellent

Table 4. Effect of full or partial replacing ZT by various sets of cytokinins.

Treatment	Kin ($mg \cdot L^{-1}$)	BA ($mg \cdot L^{-1}$)	TDZ ($mg \cdot L^{-1}$)	ZT ($mg \cdot L^{-1}$)	Proliferation rate	Average seedling height (cm)	Degree of the callus growth	Degree of seedling
1	0	0.5	0.3	0	1.24 ± 0.05g	2.1	-	**
2	0	0.5	0.3	0.1	1.56 ± 0.15ef	0.5	-	*
3	0	1	0.8	0	1.44 ± 0.23fg	1.2	-	*
4	0	1	0.8	0.1	1.56 ± 0.29ef	0.9	-	*
5	1	0	0.3	0	1.64 ± 0.21ef	0.5	-	*
6	1	0	0.3	0.1	1.50 ± 0.22fg	1.8	-	*
7	1	0.5	0.8	0	2.12 ± 0.15d	0.4	-	*
8	1	0.5	0.8	0.1	2.16 ± 0.22d	0.4	-	*
9	1	1	0	0	3.26 ± 0.18b	3.7	+	***
10	1	1	0	0.1	4.34 ± 0.21a	4.2	+	***
11	1.5	0	0.8	0	1.60 ± 0.16ef	0.3	-	*
12	1.5	0	0.8	0.1	1.50 ± 0.10fg	0.5	-	*
13	1.5	0.5	0	0	2.32 ± 0.19d	1.6	-	*
14	1.5	0.5	0	0.1	2.70 ± 0.19c	2.1	-	*
15	1.5	1	0.3	0	1.84 ± 0.21e	1.7	-	*
16	1.5	1	0.3	0.1	2.32 ± 0.22d	0.7	-	*
CK	0	0	0	1.5	4.46 ± 0.40a	4.4	+	***

Notes: means with the identical letter in the identical column aren't diverse in terms of statistics (p<0.05).The numerical results denote average ±SD. (-) indicates no callus, (+) indicates some callus and (*) indicates status of seedling growth. *: poor, **: good, ***: excellent

Effect of full or partial replacing ZT by various sets of cytokinins: Shoot proliferation varied in different combinations of cytokinin in multiplication media (Table 4). The cytokinin TDZ was found to be non-beneficial in terms of improving the proliferation rate compared with the cytokinin kin and BA. In addition, the shoots cultured in WPM containing TDZ had malformations. Of the combinations tested, 1.0 $mg \cdot L^{-1}$ Kin, 1.0 $mg \cdot L^{-1}$ BA, and 0.1 $mg \cdot L^{-1}$ ZT led to the greatest shoot proliferation rate (4.34) (Fig. 1H). However, no significant difference in proliferation rate was obtained when compared with that in intermediary added with 1.5 $mg \cdot L^{-1}$ ZT (4.46).

Discussion

At the proliferation stage of *V. dunalianum*, an efficient and stable proliferative system can be sustained only by adding the hormone ZT to the subculture medium. In agreement with our report, Meiners (2007) reported that shoots of cowberry (*Vaccinium vitis-idaea* L.) cultivar 'Red Pearl' and blueberry (*Vaccinium corymbosum* L.) cultivar 'Ozarkblue', proliferated best when nodal segments were cultivated in an intermediary added with ZT. *In vitro* propagation of *V. angustifolium* was constructed on an adjusted mossberry tissue cultivation intermediary with ZT (Debnath, 2004). Hence, ZT is highly preferred in shoot multiplication of *Vaccinium* species.

ZT is a natural cytokinin that belongs to isoprenoid cytokinins. ZT is a very expensive cytokinin compared with BA, kin, and TDZ, but it may be the only one effective in inducing a high number of shoots and sustaining growth in some species (Ostrolucká *et al*., 2004). Herein, shoots did not easily develop into multiple shoots when cultivated

on subculture intermediary with BA, kin, or TDZ. The addition of ZT is indeed helpful for shoot proliferation of *V. dunalianum*, thereby confirming that some plants have preference for cytokinin. This result may be related to the structure and metabolism of ZT. ZT is a typical representative of isoprenoid cytokinins, and it differs from aromatic cytokinins (BA and Kin) in terms of biochemistry, receptor, biological activity, and metabolism (Gajdošová *et al*., 2011). Efficient and stable proliferative systems of some members of *Vaccinium* were established by the addition of ZT or 2iP to the subculture medium (Debnath, 2007). The reason for this phenomenon may be that ZT and 2iP are naturally occurring cytokinins that have similar structures. ZT may be formed from isopentenyladenine by hydroxylation of one of terminal side chain methyl groups (Astot *et al*., 2001).

BA, which belongs to aromatic cytokinins, is a widely used and affordable cytokinin for shoot proliferation of many plants, like *Fragaria vesca* (Zhang *et al*., 2014; Koskela *et al*., 2012), *Salix pseudolasiogyne* (Park *et al*., 2008), and *Platanus acerifolia* (Bao *et al*., 2017). For some plants BA (benzyladenine) is more valid in contrast to the alternative forms of phytokinin (i.e., ZT, Kin) for shoot proliferation (Park *et al*., 2008). However, herein, the addition of 1.0-3.0 $mg \cdot L^{-1}$ BA resulted in the low multiplication rate (1.38-1.42) of *V. dunalianum.* The harmful effect of BA may be caused by the glucosylation or alanine combination, causing inactive features in terms of biology but quite steady derivants in terms of chemistry and a retarded releasing of BA from the derivants (Buah *et al*., 2010).

Similar to BA, Kin is an aromatic cytokinin (purine derivatives) that often causes similar effects on plants (Venkatachalam *et al*., 2007). In this research, the lowest proliferation rate (1.38-1.58) was acquired via 2.0-4.0 $mg \cdot L^{-1}$ Kin in WPM. Thus, cytokinins with similar structures may produce the same effect on the shoot proliferation of *V. dunalianum.* However, although BA and Kin belong to aromatic cytokinins, they still have some structural differences. For example, in contrast to BA with a benzylamno side chain, Kin has a furfurylamino side chain. Structural differences between different members of aromatic cytokinins may lead to different plant responses. For *Echinops kebericho* and other components of the Asteraceae family, Kin with weaker division ability is more valid in contrast to BA for shoot multiplication and shoot length (Enyew & Feyissa, 2018).

TDZ is synthetic cytokinin that doesn't have the purine ring seen in other amidopurine-type phytokinins, such as BA, kin, and ZT. TDZ has been shown to be better than other cytokinins for shoot multiplication of certain plants (Oosumi *et al*., 2006; Landi & Mezzetti, 2006). However, the metabolism of TDZ is extremely slow, while plant tissues completely metabolize ZT within hours of its application. TDZ has a negative potency on shoot proliferation for certain plants (Dewir *et al*., 2018). In this research, the use of TDZ as phytokinin not only failed to improve the shoot proliferation coefficient of *V. dunalianum* but also led to the appearance of malformed buds. Such discovery is in agreement with the outcomes of Cappelletti *et al*., (2016), who reported that TDZ could not be utilized as a substitution of ZT for blueberry multiplication.

In general, screening of formulations is time consuming and laborious in the process of plant tissue cultivation. In research and development of tissue culture of a plant, the basic culture medium, the characteristics of hormones, and similar literature reported by predecessors are often used to establish a tissue culture system successfully and quickly. However, hormones used are different in members of the same genus. How to quickly and efficiently screen the most suitable formulation is the problem faced by many researchers. By combining the present data with previous studies on tissue culture of *Vaccinium*, we infer that members of this family have a high preference for ZT in tissue culture. Moreover, according to our study of the replacement potency of other phytokinins (Kin, BA, and TDZ) on ZT in the shoot proliferation stage of *V. dunalianum*, members of the family have a high preference for ZT. Therefore, We suggest that ZT or its similar isoprenoid cytokinins, such as 2ip, should be given priority in tissue culture of this genus.

Conclusion

In this study, we reported the replacement effect of other cytokinins (Kin, BA, and TDZ) on ZT in the shoot proliferation stage of *V. dunalianum*. We found the high preference for ZT in shoot multiplication of *V. dunalianum*. ZT could not be completely replaced by BA, Kin and TDZ. In addition, we speculate that ZT is the most ideal cytokinin for the tissue culture of *Vaccinium* species and should be used preferentially in formula selection. This work can provide scientific guidance for screening *Vaccinium* species micropropagation formula and improve the screening efficiency in plant tissue culture. The production cost of seedlings can also be reduced to a certain extent by partial replacement of ZT, which has a high price.

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