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Research Article
Influence of Different Cytokinins on the Growth, [6]-Gingerol Production and Antioxidant Activity of *in vitro* Multiple Shoot Culture of Ginger (*Zingiber officinale* Roscoe)

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Abstract
The growth, [6]-gingerol production and antioxidant activity of ginger multiple shoots cultured in MS medium containing 1 mg L^{-1} α-naphthalene acetic acid (NAA) in combination with varying levels of different cytokinins, namely, benzylaminopurine (BAP), kinetin (KIN) and thidiazuron (TDZ) were determined. The [6]-gingerol content ranged from 125-344 µg per culture bottle, with the highest production found in ginger shoot cultures planted in MS medium containing 2 mg L^{-1} BAP. Growth was inversely correlated to [6]-gingerol production attributed to the negative effects of oxidized phenols on plant development. The antioxidant activity, as determined by FRAP assay, was highly correlated with total phenolics and [6]-gingerol content.

Key words: Cytokinin, ginger, gingerol, multiple shoots

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Zingiber officinale Roscoe, commonly known as ginger of the family Zingiberaceae, is one of the most heavily consumed dietary substances in the world (Surh *et al*., 1998). It contains several interesting bioactive constituents and possesses health promoting properties (Wong *et al*., 1999). Its major constituents are the sesquiterpene hydrocarbons, diarylheptanoids and gingerol-related compounds. The gingerol-related compounds are believed to exhibit anti-inflammatory and anti-nausea effects (Afzal *et al*., 2001). The [6]-gingerol is the predominant phenol and most important of the pungent constituents in ginger oil (Jiang *et al*., 2006). The quantity and quality of this polyphenol present in ginger can vary significantly due to different factors, such as plant genotype and cultivar, growing conditions and maturity state among others (Jeffery *et al*., 2003).

Advances in plant biotechnology has allowed the production of plant biochemicals *in vitro* such as the production of shikonin and berberine from cultured cells of *Lithospermum erythrorhizon* and *Coptis japonica* (Fujita, 1988). In general, however, the production of desired compound is absent or low in callus or cell culture but is higher in differentiated or organized tissues, e.g., hairy roots or multiple shoots. Modification of nutritional and cultural conditions to effect differentiation is often done to improve the production of target compounds. The influence of plant growth regulators on the growth and enhanced metabolite production has been shown in a number of plant species (Sudria *et al*., 2001; Prins *et al*., 2010; Coste *et al*., 2011; Rawat *et al*., 2013; Santoro *et al*., 2013; Awate and Gaikwad, 2014). Among plant growth regulators, cytokinins are known to induce multiple shoot proliferation (Zhang and Cui, 2001; Belide *et al*., 2010; Namli *et al*., 2010; Khatoon *et al*., 2014). No investigation so far had been conducted to study the influence of cytokinins on [6]-gingerol production *in vitro*. This paper is the first report regarding the influence of cytokinins on [6]-gingerol production *in vitro*, relating it to the growth and antioxidant activity of ginger multiple shoots.

MATERIALS AND METHODS

Plant materials: Mature rhizomes of *Zingiber officinale* cv. 'Imugan' were collected around the vicinity of BIOTECH, UPLB, Laguna, Philippines. They were established in the laboratory as multiple shoot cultures by aseptically planting surface-sterilized shoot apical meristems in culture bottles containing 25 mL of MS medium (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} NAA+1 mg L^{-1} BAP. Ginger shoot cultures were maintained at 25±2°C under a photoperiod of 16 h light and 8 h darkness. They were routinely subcultured at monthly intervals by separating the individual shoots of the multiple shoot clumps and transferring them in fresh similar medium.

Cytokinin treatments: Individual shoot cultures from the multiple shoot clumps were transferred to MS medium supplemented with 1 mg L^{-1} NAA in combination with varying levels (0-2 mg L^{-1}) of different cytokinins, namely BAP, KIN and TDZ. After four weeks, the growth response of the multiple shoots to various cytokinin treatments was compared by counting the number of shoots formed per culture bottle and by measuring the length of the longest shoot.

Preparation of the samples: The multiple shoots from each culture bottle were harvested, macerated and soaked in 100 mL methanol overnight. The methanolic extracts were then passed through Whatman filter paper No. 1. The filtrates were concentrated under vacuum on a rotary evaporator at ≤40°C. The residue was re-suspended in methanol and stored in the refrigerator prior to analysis of [6]-gingerol content, total phenolics content and antioxidant activity.

Quantitative analysis of [6]-gingerol content: Quantitative determination of [6]-gingerol content was performed using a reversed-phase HPLC following the methods of Cafino (2013). Separation was achieved using an Inertsil ODS-3 column (250×4.6 mm) maintained at 25°C. Mobile phase consisted of methanol:water (90:10, v/v) ran isocratically at a flow rate of 1 mL min^{-1} for 5 min. The detection wavelength was set at 282 nm (Pawar *et al*., 2010). The [6]-gingerol content per culture bottle was determined after comparison with a calibration curve using authentic [6]-gingerol (Sigma-Aldrich, St. Louis, Missouri) as standard.

Determination of total phenolics content: The total phenolics content was determined following the methods of Patil *et al*. (2009). Briefly, 1 mL of methanol extract was added to 10 mL deionized water and 1 mL Folin-Ciocalteau phenol reagents. After 5 min, 2 mL of 20% sodium carbonate was added to the mixture and kept in total darkness for 2 h. The absorbance was measured spectrophotometrically at 750 nm. Total phenolics content per culture bottle, expressed as mg gallic acid equivalent, was calculated after comparison with a calibration curve using gallic acid as standard (Kim *et al*., 2003).

Determination of antioxidant activity: The antioxidant activity was determined following the Ferric Reducing Antioxidant Power (FRAP) assay of Benzie and Strain (1996). The FRAP reagent contained 2.5 mL of 10 mM TPTZ (2,4,6-tripyridyl-1,3,5-triazine) in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL 0.3 M acetate buffer pH 3.6. Forty microliters of methanol extract were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The reaction mixture was incubated at 37°C for 10 min and absorbance was measured spectrophotometrically at 593 nm. The antioxidant activity per culture bottle, expressed as µg $FeSO_4$ equivalent, was calculated after comparison with a calibration curve using $FeSO_4$ as standard, which was linear between 10 and 380 µg mL^{-1}.

Statistical analysis: Two-factor factorial experiments were performed in a Completely Randomized Design (CRD). Data were subjected to analysis of variance (ANOVA) and comparison among treatment means was done using Tukey's HSD multiple comparisons test. The p-value of <0.05 was regarded as significant.

Time and place of the study: This study was conducted from October, 2013 to April, 2014 at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, Collage, Laguna, Philippines.

RESULTS AND DISCUSSION

Influence of cytokinin on growth of ginger multiple shoots: The number of shoots per culture bottle induced by KIN, BAP and TDZ at varying levels after four weeks is shown in Fig. 1. Supplementation with 1 mg L^{-1} KIN resulted in the highest number of induced shoots, with an average of 5 shoots per culture bottle. At same concentrations, BAP and TDZ were less effective than KIN in inducing multiple shoot formation. BAP was most effective when used at 0.5 mg L^{-1}, which induced an average of 3.3 shoots per culture bottle. TDZ, on the other hand, was most effective when used at 2 mg L^{-1}, which induced an average of 3.7 shoots per culture bottle. However, shoot induction by BAP and TDZ, at any tested concentrations, was not significantly different to the treatment without cytokinin which averaged 2.9 shoots per culture bottle.

Figure 2 shows the influence of KIN, BAP and TDZ on the length of ginger multiple shoots. At same concentrations, KIN was the best cytokinin to produce long shoots, followed by BAP and then by TDZ. However, increasing the concentration

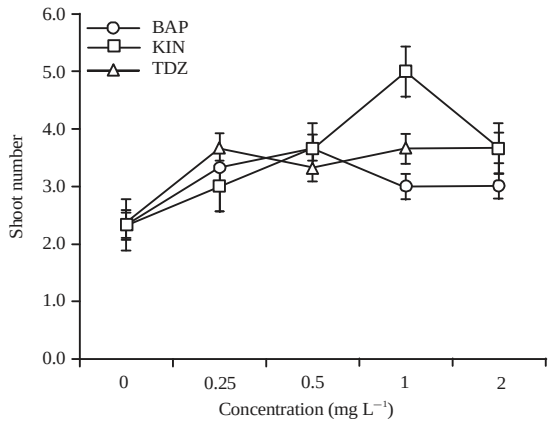

Fig. 1: Effect of the different cytokinins at varying concentrations on the number of induced shoots of *Zingiber officinale* four weeks after transfer

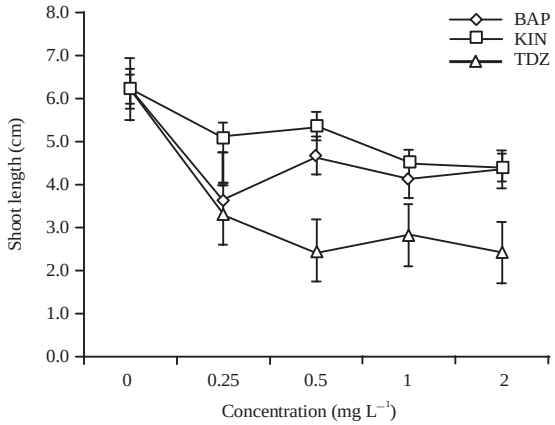

Fig. 2: Effect of the different cytokinins at varying concentrations on the length of induced shoots of *Zingiber officinale* four weeks after transfer

of any of the three tested cytokinins resulted to a decreasing shoot length. In fact, the longest shoots were observed in ginger grown in MS medium without any cytokinin. Generally, plants cultured on media supplemented with relatively high levels of cytokinin resulted to increased adventitious shoot regeneration (Erisen *et al*., 2011) since cytokinins are responsible for cell division and for stimulating shoot initiation and growth. With the induction of multiple shoot formation, many shoots compete with the limited nutrients available in the culture medium. Hence, ginger multiple shoots cultured in MS medium with higher cytokinin were shorter than those cultured in MS medium with lower or without cytokinin.

[6]-gingerol content: As shown in Fig. 3, [6]-gingerol content per culture bottle ranged from 125-344 µg for multiple

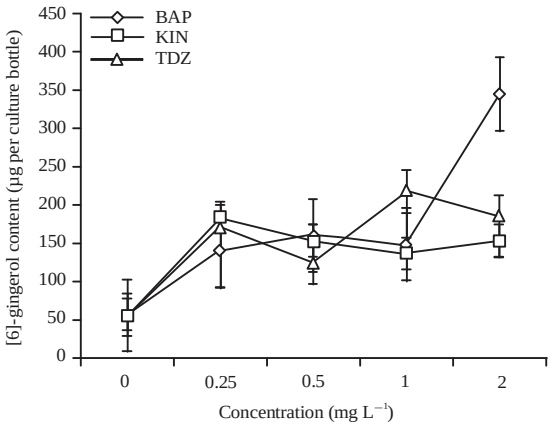

Fig. 3: Gingerol content of ginger multiple shoots cultured in MS medium supplemented with varying levels of different cytokinins

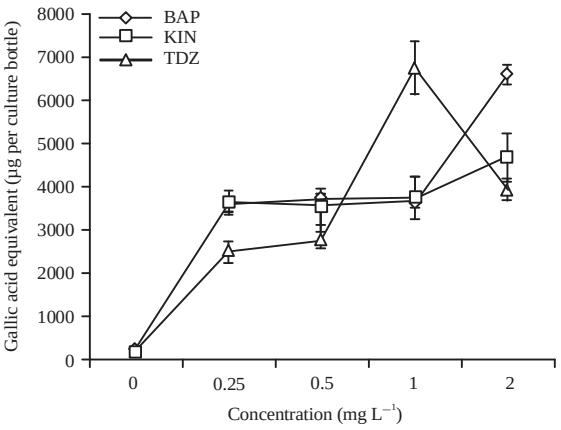

Fig. 4: Total phenolics content, expressed as µg gallic acid equivalent per culture bottle, of ginger multiple shoots cultured in MS medium supplemented with varying levels of different cytokinins

shoots cultured in medium supplemented with a cytokinin. This was significantly higher than those of multiple shoots cultured in medium without cytokinin, i.e., 57 µg per culture bottle, suggesting that the amount of [6]-gingerol in ginger multiple shoots increased in the presence of a cytokinin. Similar amount of [6]-gingerol production was observed when BAP, KIN and TDZ were used at 0.25-1 mg L^{-1}. When these cytokinins were further increased to 2 mg L^{-1}, no increase in [6]-gingerol production was observed, except for 2 mg L^{-1} BAP which showed the highest [6]-gingerol production of 344 µg per culture bottle.

Total phenolics content: Phenolic compounds were commonly found in plants and have been reported to have several biological activities including their potential as antioxidants and free radical scavengers (Robards *et al*., 1999). In ginger, the phenolics are important components and some of its pharmacological effects could be attributed to the presence of these valuable compounds (Ghasemzadeh *et al*., 2010).

As shown in Fig. 4, the total phenolics content of samples obtained from ginger multiple shoots grown in medium with 0.25-2 mg L^{-1} cytokinin was in the range of 2529.0-6782.2 µg per culture bottle. The highest total phenolics content was found in multiple shoots grown in medium with 1 mg L^{-1} TDZ (6782.2 µg per culture bottle) and also in medium with 2 mg L^{-1} BAP (6620.8 µg per culture bottle). The TDZ, when used at 2 mg L^{-1}, was inhibitory to the production of total phenolics. A similar observation was reported by Rewatkar (2012) attributing this to the toxic effects exhibited by TDZ at concentrations above 1 mg L^{-1}. The total phenolics content in samples treated with a cytokinin was higher than those treated with 1 mg L^{-1} NAA alone which had 231.1 µg per culture bottle.

Determination of antioxidant activity: As mentioned by Tanaka *et al.* (1988), there is a direct correlation between antioxidant capacity and reducing power of plant extracts. The FRAP method measures the ability of antioxidant to reduce Fe^{3+} to their respective lower valency state. The $FeSO_4$ equivalent is the amount of Fe^{2+} present in the reaction mixture that is produced by the action of the antioxidant on the ferric-TPTZ complex. The $FeSO_4$ equivalents of methanol extracts of ginger multiple shoots grown in medium with a cytokinin were found within the range of 1429-2860 µg per culture bottle with the maximum antioxidant activity found in plants supplied with 2 mg L^{-1} BAP (Fig. 5).

Relationships among shoot growth, [6]-gingerol content, total phenolics content and antioxidant activity: Table 1 shows the relationships among shoot growth, [6]-gingerol content, total phenolics content and antioxidant activity. A positive relationship among [6]-gingerol content, total phenolics content and the antioxidant activity can be observed for ginger multiple shoots. It supports the idea that the phenolic hydroxyl groups present in plant antioxidants have redox properties allowing them to act as a reducing agent and a hydrogen donor (Shahidi *et al*., 1992). On the other hand, relationships between shoot growth and total phenolics content or shoot growth and [6]-gingerol content are rather negative. The same can be observed for the relationship between shoot growth and antioxidant activity.

Table 1: Pearson correlations among different shoot growth parameters, antioxidant capacity, total phenolics content and [6]-gingerol content of *Zingiber officinale*

Parameters	Total phenolics	Antioxidant capacity	Shoot height	Shoot number
[6]-gingerol	0.8032*	0.9027***	0.2590	0.1729
Total phenolics		0.8781**	0.5399	0.0018
Antioxidant capacity			0.5201	0.0548

*Significance level at p<0.05, **Significance level at p<0.01, ***Significance level at p<0.001

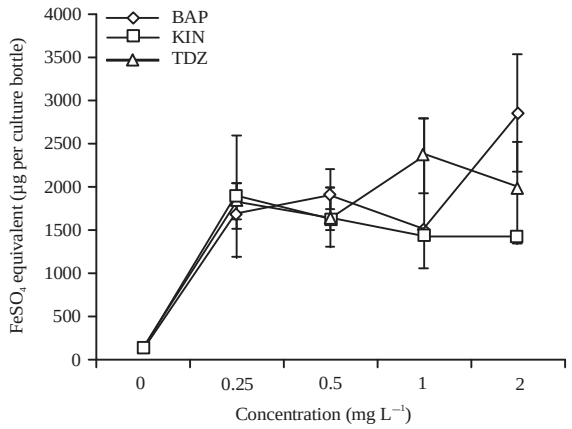

Fig. 5: Antioxidant activity, expressed as µg $FeSO_4$ equivalent per culture bottle, of ginger multiple shoots cultured in MS medium supplemented with varying levels of different cytokinins

Some bioactive compounds present in medicinal plant possessed high total antioxidant activity, which was due to the presence of phenolics (Kim *et al*., 2011; Do *et al*., 2004; Zheng and Wang, 2001). The strong correlation between [6]-gingerol content and its antioxidant potential coincides with the relationship reported by Pawar *et al*. (2010) for EMS-treated ginger rhizomes. This supports the well-established fact that the metal-binding properties of phenolic compounds offered antioxidant action by encapsulation of a pro-oxidant iron species, which generates hydroxyl radical species through the Fenton reaction (Ferrali *et al*., 1997) and that gingerols exert compatible inhibitory activity against Fenton-generated hydroxyl radicals (Dugasani *et al*., 2010).

The negative relationship between phenolic compounds and shoot growth was expected. Phenols are synthesized by plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances especially oxidized phenols, generally affect *in vitro* development negatively (Arnaldos *et al*., 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen *et al*., 1999). It seems, therefore, that the shorter shoot length observed in ginger cultures supplemented with high cytokinin treatment was not due solely to nutrient competition but could also be attributed to high phenolics production.

CONCLUSION

Based on the above results, ginger multiple shoots treated with cytokinins exhibited significantly higher [6]-gingerol production than the treatment without cytokinin. Our results also showed that 2 mg L^{-1} BAP+1 mg L^{-1} NAA is the best hormone combination for [6]-gingerol production and antioxidant activity.

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