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Arda Acemi

Growth enhancing effects of banana homogenate on a glucomannan-rich orchid species: *Serapias vomeracea* (Burm.f.) Briq.

Wachstumsfördernde Wirkung von Bananenhomogenisat auf eine Glukomannan-reiche Orchideenart: *Serapias vomeracea* (Burm.f.) Briq.

Abstract

Glucomannan is a plant-sourced polymer used mainly in the food, pharmaceutical, and cosmetic industries. The increasing demand for the polymer makes researchers put effort into finding agriculturally sustainable and eco-friendly production methods for the source plants. The root systems of terrestrial orchids are rich sources of glucomannan. The objective of this research was to evaluate the concentration-dependent (10, 30, and 50 g L^{-1}) effects of banana homogenate (BAN) on *in vitro* development of a glucomannan-rich orchid species, *Serapias vomeracea*. The control medium gave the highest seed germination rate. In contrast, the addition of 10 g L^{-1} BAN into the medium either in the presence or absence of sucrose gave statistically the same germination results with the control. However, all the BAN concentrations tested triggered protocorm formation significantly better in the absence of sucrose in the medium. The maximum mean shoot and root lengths, and root number were recorded after BAN treatments at 30 g L^{-1} concentration. The tuber formation and development were shown to be enhanced gradually due to BAN treatments. The successful tuberization in *S. vomeracea* roots was achieved after 50 g L^{-1} BAN, which gave more tubers in numbers with larger diameters. This study suggested that BAN could be used for tuber production for industrial demand. In the large-scale sustainable cultivation of tuberous orchids, synthetic plant growth regulators might be replaced by this natural additive. Also, BAN should be evaluated in *in vitro* propagation studies on Mediterranean terrestrial orchids as a substitute for sucrose.

Key words: Long-lipped *Serapias*, Natural additives, Orchid culture, Rhizogenesis, Tuberization

Zusammenfassung

Glukomannane sind pflanzliche Polymere, die hauptsächlich in der Lebensmittel- sowie in der pharmazeutischen und kosmetischen Industrie genutzt werden. Auf Grund der steigenden Nachfrage wurden in den letzten Jahren vermehrt wissenschaftliche Bemühungen unternommen, um eine landwirtschaftlich nachhaltige und ökologische Kultivierung glukomannanreicher Pflanzen zu entwickeln. Das Wurzelgeflecht der terrestrischen Orchideen ist eine reichhaltige Quelle für Glukomannan. Das Ziel dieser Arbeit war es den konzentrationsabhängigen Effekt von Bananen-Homogenat (BAN) auf die In-vitro-Entwicklung der glukomannanreichen Orchideenart *Serapias vomeracea* zu ermitteln. Die Keimungsrate war bei Nutzung des Kontrollmediums am höchsten. Die Zugabe von 10 g L^{-1} BAN zum Medium, sowohl mit als auch ohne Zugabe von Saccharose, führte allerdings zu einer statistisch sehr ähnlichen Keimungsrate. Allerdings führte die Zugabe von BAN in Abwesenheit von Saccharose in sämtlich getesteten Konzentrationen zu einer signifikant

Affiliation
Kocaeli University, Faculty of Arts and Sciences, Department of Biology, Kocaeli, Turkey

ORCID
Dr. Arda Acemi https://orcid.org/0000-0003-0270-8507

Correspondence
Dr. Arda Acemi, Kocaeli University, Faculty of Arts and Sciences, Department of Biology, 41001 İzmit, Kocaeli, Turkey, Tel.: +902623032170, e-mail: arda.acemi@kocaeli.edu.tr

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vermehrten Bildung von Protokormen. Die durchschnittlichen Spross- und Wurzellängen sowie die Anzahl der Wurzeln wurden bei einer BAN Konzentration von 30 g L^{-1} evaluiert. Die Knollenbildung und -entwicklung waren bei Zugabe von BAN leicht verbessert. Die Anzahl der Wurzelknollen von *S. vomeracea* und ihr Durchmesser waren bei Zugabe von 50 g L^{-1} BAN erhöht. Diese Studie legt nahe, dass BAN in der Produktion von glukomannanreichen Wurzelknollen für industrielle Zwecke genutzt werden könnte. Synthetische Pflanzenwachstumsregulatoren könnten im Zuge des nachhaltigen Anbaus von knollenbildenden Orchideen durch BAN ersetzt werden. BAN sollte weitergehend hinsichtlich seiner Eignung als Ersatz für Saccharose bei Studien zur In-vitro-Vermehrung mediterraner Orchideen untersucht werden.

Stichwörter: Lang-lippige *Serapias*, natürliche Zusätze, Orchideenanbau, Wurzelbildung, Wurzelknollenbildung

Introduction

The terms „natural additives“ or „natural substances“ in plant tissue culture describe the medium components with natural origins that contribute to plant growth but not categorized in any plant growth regulator (PGR) class (Molnár et al., 2011). The most frequently used natural additives are reported as banana homogenate (BAN), coconut water, and extracts of malt, potato, and yeast (George et al., 2008). Among these natural substances, BAN, which is mostly used in orchid cultures, is preferred because of its nutritional properties. In tropical orchid cultures, the addition of BAN into the medium has been reported to increase shoot and root growth, and to enhance the seed germination and protocorm-like body formation rates (Vyas et al., 2009; Shekarriz et al., 2014; Parthibhan et al., 2015). However, the effects of BAN on Mediterranean terrestrial orchids have not been elucidated comprehensively yet. These orchids are harvested for their glucomannan (GM)-rich tubers, sometimes illegally, during their flowering seasons and marketed at high prices, to be used mainly in the food industry. Therefore, over-collection of the orchids for commercial purposes could be considered one of the reasons behind their limited populations in the future (Acemi et al., 2019). In this context, a sustainable and eco-friendly method should be implemented for the production of GM-source orchids.

Serapias vomeracea is a widespread orchid species with a distribution from Turkey to the Iberian Peninsula. The GM content of its tubers up to 30% renders it attractive for the food and pharmaceutical industries since this polymer has high hydrophilic and probiotic properties which make it a natural gelling agent, a food thickener, and a therapeutic agent (Sood et al., 2008; Acemi et al., 2019). In the previous studies, the effects of chitosan and different media formulations on the *in vitro* propagation of *S. vomeracea* were reported (Acemi and Özen, 2019; Acemi, 2020). However, the effects of natural additives on *in vitro* propagation of *S. vomeracea* have not been studied yet. Therefore, this study aimed to compare the concentration-dependent effects of BAN, a commonly-used natural additive in orchid cultures, on the *in vitro* development of *S. vomeracea*. Comparison of the effects of BAN treatments on *in vitro* growth parameters of a widespread and commercially important model orchid species such as *S. vomeracea* would highlight the favorable effects of this natural culture medium supplement. The findings would lead to the sustainable horticultural development of tuberous orchid species for commercial use. In addition, in case of a possible enhancement in the orchid's development after BAN treatment, synthetic plant growth regulators might be replaced by this natural additive for the large-scale sustainable cultivation of tuberous orchids.

Materials and methods

In vitro culture establishment

The Directorate of the Aegean Agricultural Research Institute (Menemen, İzmir, Turkey) supplied the seeds of *Serapias vomeracea* for the experiments. The seeds were then stored in a dry and dark place at 4°C until they were used. The tetrazolium (2,3,5-Triphenyltetrazolium chloride; TTC) test was used to determine the seed viability rate in *S. vomeracea* (Acemi and Özen, 2019). The seeds were disinfected following the previously established method (Acemi, 2020).

Knudson C (KN) medium fortified with BAN at three concentrations (10, 30, and 50 g L^{-1}) was employed in the experiments (Knudson, 1946). Banana fruits were purchased from a local market in Kocaeli, Turkey. The peeled and sliced bananas were lyophilized for 48 h. The completely dried banana slices were powdered using mortar, and this final product was named banana homogenate. The homogenate was kept in the same environmental conditions as the seeds. KN medium without BAN was used as the control. The effects of BAN on seed germination and protocorm formation were tested either in the presence (BAN + Sucrose) or absence (BAN − Sucrose) of sucrose in the medium. The most promising medium was selected and employed in further experiments. The medium was supplemented with 20 g L^{-1} sucrose when it was employed. Phytagel at 3.5 g L^{-1} concentration was used to solidify the medium, and the pH of the medium was set to 5.6 before autoclaving. One milligram of disinfected seed was inoculated onto the culture medium in Petri dishes. After the incubation period, the developed protocorms were transferred into culture vessels containing the same medium. The cultures were held at 23 ± 1°C with an illumination of 60 µmol m^{-2} s^{-1} photosynthetic photon flux intensity under 16/8 h light/dark photoperiod.

Data collection and statistical analysis

The seed germination rate was determined at the end of the 45th day of the incubation period, while the growth of

protocorm was assessed after the 90th day of the incubation period. The organ development was evaluated at the end of the 180th day of the incubation period, which began after the transition of protocorms to culture vessels. Seeds were counted and divided into six separate germination phases shown in Table 1 as suggested by Yamazaki and Miyoshi (2006).

The percentages of germination were determined using the following formula (1).

(1) Germination percentage (%) = Σ Seed number (Stage 2–5) × 100/Σ Seed number (Stage 0–5)

Approximately 180–200 individual seeds are found in 1 mg of *S. vomeracea* seed. Five protocorms were cultivated in each culture vessel, and 15 protocorms were employed in each repeat. Each experiment was carried out in triplicate. The data were represented as mean ± standard deviation (SD). Means were compared using either Duncan's Multiple Range Test (DMRT) or Tukey's Honestly Significant Difference (HSD) Test at a significance level of $p < 0.05$. The statistical analysis was performed through IBM SPSS software, version 22. The hierarchical clustering was done using the normalized data. Euclidian distance and unweighted pair group method with arithmetic mean (UPGMA) was used for cluster analysis. The clustering heatmap was created through BioVinci data visualization software, version 1.1.5.

Results

Seed germination and protocorm formation

The result of the TTC test showing the rate of viable *S. vomeracea* seeds was found 54.67 ± 4.16%. The seed germination rate was found 58.77 ± 5.51% from the control medium. The increasing BAN concentrations reduced the seed germination rate gradually, in the presence of sucrose in the medium. A more sharp decrease was found in the same parameter when sucrose was removed from the medium. However, regardless of the presence of sucrose in the medium, the minimum concentrations of BAN in the medium gave statistically the same germination results with the control (Fig. 1a).

In the experiments, the simultaneous presence of sucrose and BAN in the medium did not make any statistically significant effect on protocorm formation compared to the control. However, a dramatic effect was found when the sucrose was removed from the medium (Fig. 1b). The BAN concentrations triggered the protocorm formation between 9 and 10-fold in comparison with the control, which gave the protocorm formation rate of 7.45 ± 0.25%. Further experiments on the root and shoot development were carried out using the same medium without sucrose but supplemented with BAN concentrations.

Effects of BAN on plant development

The treatments of 30 and 50 g L^{-1} BAN increased the shoot length in comparison with the control (Fig. 2a). The medium with 10 mg L^{-1} BAN gave the statistically same result with the control. The same trend was also found for the root elongation in *S. vomeracea*. The medi-

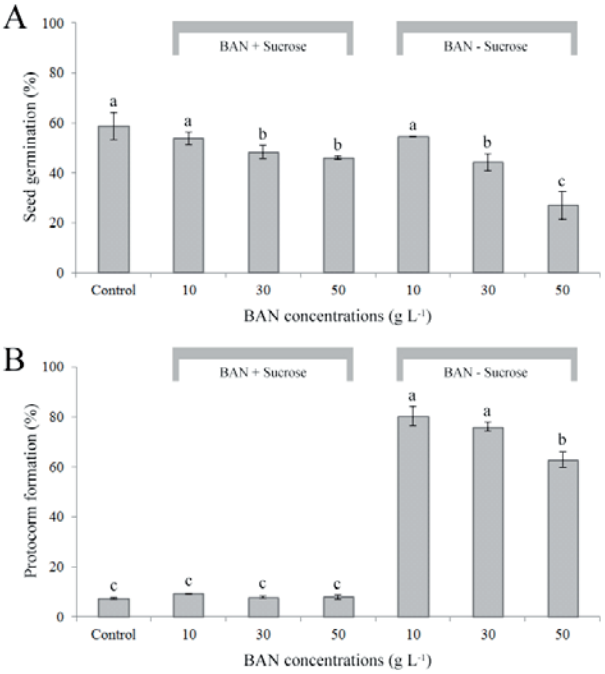

Fig. 1. The effects of banana homogenate (BAN) on the seed germination (A) and protocorm formation rates from the germinated seeds (B) in *Serapias vomeracea*. Data represent mean ± SD. Means having the same superscript letters were not significantly different by Duncan's multiple range test ($p < 0.05$).

Table 1. Stages of seed germination according to Yamazaki and Miyoshi (2006)

Germination stage	Indicators
Stage 0	'No germination' stage. No growth of embryo occurs.
Stage 1	'Pre-germination' stage. Embryo swells to fill the seed coat.
Stage 2	'Germination' stage. Embryo emerges from the seed coat.
Stage 3	'Protocorm' stage. Embryo is completely discharged from the seed coat.
Stage 4	'Rhizoid' stage. Rhizoids are formed on the protocorm surface.
Stage 5	'Shoot' stage. Shoot is differentiated from the protocorm.

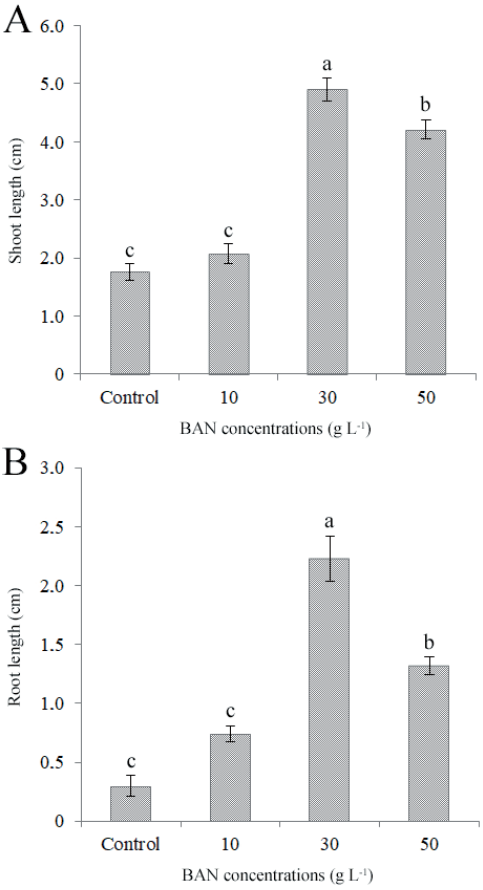

Fig. 2. **The effects of banana homogenate on the mean shoot (A) and root (B) lengths in *Serapias vomeracea*.** Data represent mean ± SD. Means having the same superscript letters were not significantly different by Tukey's honestly significant difference test ($p < 0.05$).

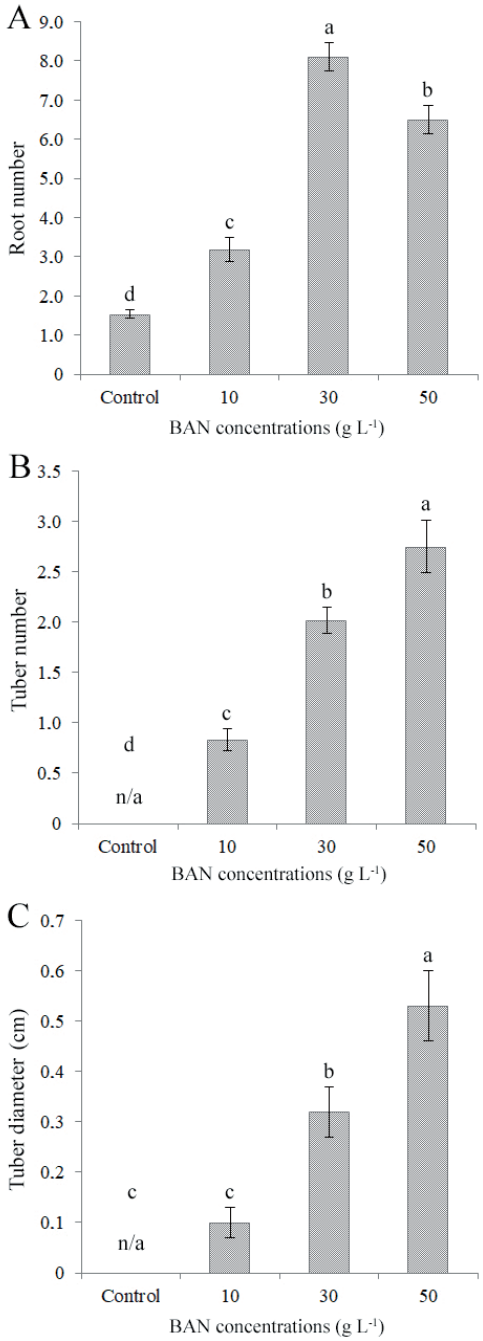

Fig. 3. **The effects of banana homogenate on the mean root (A) and tuber (B) numbers, and tuber thickness (C) in *Serapias vomeracea*.** Data represent mean ± SD. Means having the same superscript letters were not significantly different by Tukey's honestly significant difference test ($p < 0.05$).

um with 30 g L^{-1} BAN gave the maximum mean root length (Fig. 2b). However, the leaf browning occurred in the same medium.

The BAN treatments increased the rhizogenic response in *S. vomeracea*. The medium with 30 g L^{-1} BAN induced the maximum mean root number (Fig. 3a). Different growth patterns were found for the amount and diameter of the tuber, although no production of the tuber was detected in the control group (Fig. 3b). The mean tuber number and diameter increased concentration-dependently in the presence of BAN in the medium. The medium with 50 g L^{-1} BAN gave the best tuberization result. Also, BAN concentration in the medium was effective in tuber growth. The thickest tubers were found in the plants grown in the medium with 50 g L^{-1} BAN (Fig. 3c).

The general appearance of the plants after treated with different concentrations of BAN is given in Fig. 4.

Cluster analysis on the developmental data

Similarity associations between the results of the BAN concentrations evaluated on *S. vomeracea* are presented in a UPGMA tree based on developmental data (Fig. 5). The tested concentrations were grouped into two main clusters. The media containing 30 and 50 g L^{-1} BAN were placed in the same cluster while the control and the medium with 10 g L^{-1} BAN were located in the other cluster.

Discussion

As one of the organic additives, BAN is used in plant tissue cultures since it has a rich nature in natural vitamins, phenols, fibers, hormones, and proteins (Utami et al.,

Fig. 4. The concentration-dependent effects of banana homogenate on *in vitro* development of *Serapias vomeracea* after 270 days following the seed sowing. The plant samples in the figure are lined left to right as „Control, 10, 30 and 50 g L^{-1} of banana homogenate treatments". The scale bar represents 1 cm.

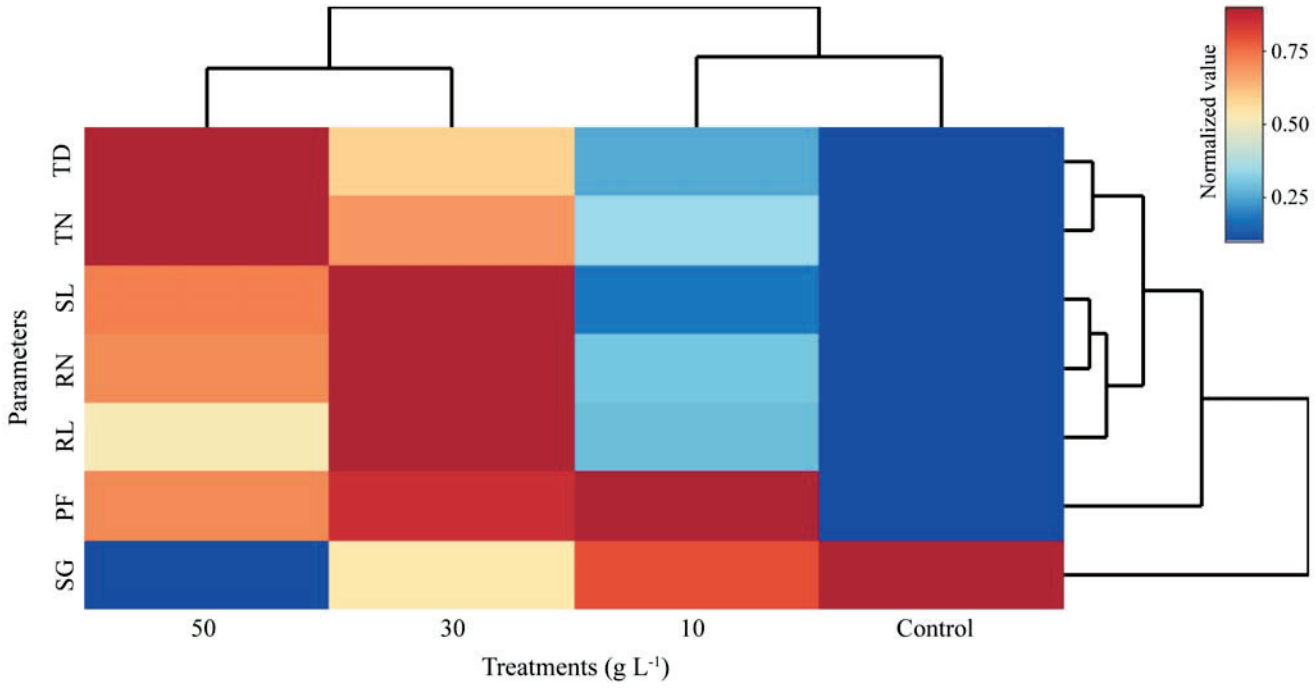

Fig. 5. Hierarchical clustering heatmap-based comparison of the developmental data. SG: seed germination, PF: protocorm formation, SL: shoot length, RL: root length, RN: root number, TD: tuber diameter, TN: tuber number

2019). This complex structure, which consists of a wide range of organic nutrients and growth factors, has yet to be fully identified. However, this study has shown that BAN exhibits different effects during the germination, protocol formation, and organ development stages of the plant due to the change in the nutritional needs of the plant.

The carbon sources used in plant tissue cultures have been shown to influence plant cell, tissue, and organ development (Roycewicz and Malamy, 2012). Sucrose has been considered the primary carbon source among the many available ones, such as mannitol, fructose, glucose, maltose, and lactose (Neto and Otoni, 2003). However, the germination results in this study showed that BAN at lower concentrations in the medium has the potential to be used as a substitute for sucrose. High protocorm formation levels following BAN treatment in the absence of sucrose suggested that BAN and sucrose together in the medium might reduce the osmotic potential of the culture medium, which could limit water and nutrient uptake. Sucrose is also completely or partly substituted by another carbon source in some experiments as it may induce hypoxia and ethanol accumulation in plant cells (Yaseen et al., 2013). Therefore, these discussions indicate the changes in the nutritional needs of the plant in every developmental stage. The highest germination rates were previously recorded (89.91%) in *S. vomeracea* cultured on the Orchidmax medium with activated charcoal and 2 mg L^{-1} zeatin (Bektaş and Sökmen, 2016). In another study, the authors suggested that simplicity of the KN might be an advantage to modify it according to the nutritional needs of *S. vomeracea* seeds for better plant development (Acemi and Özen, 2019). Also, the KN medium supplemented with chitosan polymer at 5 mg L^{-1} concentration was shown to induce a germination rate of 74.97 ± 3.62% in *S. vomeracea* seeds (Acemi, 2020). However, our study has shown that the introduction of 10 g L^{-1} BAN instead of sucrose to the KN medium with no PGRs or additives, such as activated charcoal, may give a protocorm formation ratio over 80% in *S. vomaercea*. Furthermore, the addition of BAN in *Renanthera imschootiana* Rolfe cultures has also been reported to increase protocorm-like body (PLB) formation (Wu et al., 2014).

The promotive effects of BAN on organogenesis and *in vitro* development have been previously reported in several species. Daud et al. (2011) reported increased shoot lengths in *Celosia* L. cultures treated with BAN. In *Cymbidium pendulum* (Roxb.) Sw., robust shoot and root formation were recorded after 50 g L^{-1} BAN treatment (Kaur and Bhutani, 2012). A similar favorable effect of BAN was also reported for total protein, carbohydrate, and chlorophyll amounts in *Pogostemon cablin* (Blanco) Benth. (Swamy et al., 2014). Also, Parthibhan et al. (2015) found that 3 g L^{-1} banana pulp used in the medium in *Dendrobium aqueum* Lindl. culture increased the number and length of shoots. However, there is no report available in the current literature about the effects of natural additives on *S. vomeracea* cultures. These beneficial effects of BAN might be because banana fruits are rich in K, Mg, Cu, Mn, vitamin C, and vitamin A (Wall, 2006). Despite its abundant mineral and vitamin content, BAN can act as a buffer in the medium to stabilize the pH which is a critical factor that may influence many functions such as nutrient uptake, cellular pH adjustment, rooting and cellular growth in plants (Lager et al., 2010; Chen et al., 2014). The plants incubated for extended *in vitro* culture periods may affect the medium pH since *in vitro* nutrient absorption is a function of ion exchange, which may lead to an acidic or alkaline medium pH (George et al., 2008). Furthermore, orchids are known to release phenolics *in vitro*, which may cause browning of plant tissues and pH fluctuations when accumulated in the culture medium (Yam and Arditti, 2017). Therefore, the concentration-dependent success of BAN on *in vitro* tuberization of *S. vomeracea* might be attributed to its pH stabilizing effect.

Conclusions

In the presence of sucrose, banana homogenate used at higher concentrations than 10 g L^{-1} in the culture medium reduced seed germination rate but did not affect the protocorm formation rate in *S. vomeracea*. However, banana homogenate triggered protocorm formation regardless of its concentration when sucrose was absent in the culture medium. The root development was significantly enhanced when banana homogenate was used at higher concentrations than 10 g L^{-1} in the culture medium. The highest concentration of BAN induced the formation of well-developed tubers. The clustering heatmap indicated that BAN concentrations higher than 10 g L^{-1} show distinctive effects than the other treatments. This study showed that BAN should be used instead of sucrose in *in vitro* propagation studies on *Serapias* L. species. Also, synthetic plant growth regulators may be substituted by BAN in the eco-friendly and sustainable production of *Serapias* orchids. However, it should be noted that the possible variations in chemical compositions of the fruits of different banana cultivars may alter their effects on the orchid cultures. The promising tuberization results reached in this study would contribute to the *in vitro* production of orchid tubers. Also, the effects of BAN treatments should be tested with *in vivo* experiments to produce orchid tubers for the industrial demand.

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Conflicts of interest

The author declares no conflicts of interest.

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