International Journal of Horticultural Science and Technology
Journal homepage: http://ijhst.ut.ac.ir

Effect of Cytokinin Type and pH Level on Regeneration of Ginger *in vitro*

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ARTICLE INFO

Article history:

Received: 8 April 2021,
Received in revised form: 3 August 2021,
Accepted: 7 November 2021

Article type:

Research paper

Keywords:

Ginger,
Micro-propagation,
Shoot-tip culture,
Regeneration,
Cytokinin

ABSTRACT

Ginger is unfertile species that usually failed to set seed. Cultivation of this plant using its economic part (rhizomes) is an unprofitable method, which negatively affects its supply in the market. Therefore, this study aimed to maintain the local Yemeni ginger variety, and facilitate its propagation by tissue culture technique, using fresh rhizome buds in a semi-rigid culture medium. To do so, two experiments were carried out, the first one was based on completely randomized block design with four replicates to determine the optimum pH levels (including 5.6, 5.8, 6.0) for the shoot and root formation. The result showed that the 5.8 is the optimum pH level of the medium. Second experiment was designed as two factors, type of cytokinin [6-benzylaminopurine (BAP) and Kiniten (Kin) (N6- furfuryladenine)] and ii) Cytokinin concentrations (0, 1, 2 and 3 mg L-1). The MS basal medium supplemented with above types and concentration of cytokinin with 1.0 mg L-1 IBA and 3% sucrose (BAP and Kinetin) The explants cultured on Murashige and Skoog's (MS) medium supplemented with cytokinin with four replicates. The result showed that the explants cultured on MS basal medium supplemented with 2.0 mg L-1 Kin + 1.0 mg L-1 IBA give the highest rate of shoot multiplication, shoot length and root number. In conclusion, the result obtained from this study might help to manage the propagation of ginger.

Introduction

Ginger (*Zingiber Officinale* Rosc.) is the most important spice plant in the Zingiberaceae family. Ginger production for the extraction of its active compound is oleoresins and essential oils, as well as the direct use of rhizomes for culinary purposes (Ayenew et al., 2012). In Yemen ginger is traditionally used as juice or as an additive to a coffee beverage. It is highly appreciated for its medicinal properties, such as anti-inflammatory, antifungal, analgesic and antioxidant content (Ozaki et al., 1991; Kishore and Dwivedi, 1992). Ginger rhizome uses post-natal treatment, swelling, rheumatism, application of joint pain, intestinal disturbance, and numbness of the feet (Sirirugsa, 1999). Owing to the universal outbreak of COVID-19 virus, ginger consumption achieve more interest. Ginger helps to alleviate the severe symptoms of COVID-19 positive patients and reduce the recovery time in those patients (Zahid et al., 2021). The total world production and cultivated area of ginger have sharply increased from 1992 to 2020, where China and Thailand were the major exporting countries in 1998-2000. Furthermore, importing and exporting quantity (ton) in Yemen has stimulated as 6780 and 137 tons in 2013 FAOSTAT (2013).

Overall, the propagation of plants makes up the fi rst stage of the horticultural economy by increasi

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DOI: 10.22059/IJHST.2021.321158.454

ng the cultivated area. Tissue culture techniques can be beneficial for quick clonal propagation an d production of disease-free plants with complet ely similar to their mother plants (Eshghi et al., 2 020). It has also been used effectively to protect i mportant and endangered horticulture plants (B hattacharyya and Kumaria 2014; Naderi et al. 20 21).

Ginger does not produce seeds as it is an unfertili zed species. It regularly propagates vegetatively via its rhizomes (Nair 2019). Using ginger rhizo mes as a raw material for the next crop productio n is a non-economical method, and negatively aff ects its supply to the marketplace (Zahid et al., 2 021). The suitable material for plantlet formatio n of ginger *in vitro* has been previously reported, which could be propagation by axillary buds, spr outing buds and shoot tips isolated from the rhiz omes (Doraiswamy et al., 1983; Balachandran et al., 1990; Olivier, 1996).

The level of pH plays an important role in the ma nipulation or management of the explant inducti on outcome. Abdul Aziz et al. (2012) demonstrat ed that the optimal pH of the medium for sprout f ormation is 5.5-6.0. Meanwhile, regeneration of s hoot buds requires the pH level of about 5.7 or 5. 8 (Rout and Das, 1997).

Plant growth regulators are one of the most signi ficant components of the growth and developme nt medium in tissue culture techniques, wherein the cytokinin is the most important of these regu lators because of its wide roles. The medium sup plemented with different types and concentratio ns of the hormone for vegetative and rooting gro wth of ginger has yielded different results. Previo us studies showed that the BAP and NAA combin ations were the most effective for the growth of s hoots in ginger (Sakamura et al., 1986; Charlwoo d et al., 1988; Sakamura and Suga, 1989).

Sharma and Singh (1997) reported that, the high -frequency *in vitro* multiplication of disease-free clones and successful transferring plantlets of gi nger (*Zingiber Officinale* Rosc.) obtained by cultu ring small and active buds of ginger on MS mediu m supplemented with 2 mg L^{-1} Kin and 20 g L^{-1} s ucrose. Nirmal (1997) tested various explants of ginger on MS basal medium supplemented with c ytokinins (BAP and Kin) and auxins (NAA and 2, 4-D), the medium with 4 mg L^{-1} NAA and 4 mg L^{-1} BAP gave positive response in inducing multiple shoots and roots. Ayenew et al. (2012) found tha t 2 mg L^{-1} BA + 1 mg L^{-1} Kin was better than othe r combinations on shoot number of ginger, and N AA alone developed numerous longer roots.

Behera and Santilata (2009) reported that expla nts grown on a basal MS medium containing 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA had the highest rate of ginger stem propagation. Rooting of ginger *in vitro*, was better in the basal MS medium supple mented with 2.0 mg L^{-1} NAA, and medium with 4 mg L^{-1} BAP + 3 mg L^{-1} Kin + 1 mg L^{-1} IAA caused t he best performance, whereas, MS medium with 2 mg L^{-1} IBA + 2 mg L^{-1} NAA was very effective on root initiation (Hossain et al., 2010). Eshghi et al. (2020) found that 5.4 µM NAA or 8.9 µM IBA is t he most effective treatment for rooting of *Passifl ora edulis* sprouts.

According to the aforementioned studies, the cur rent investigation aimed to develop a reliable pro tocol for the *in vitro* propagation of the local Yem eni ginger variety in order to: i) reveal the best o ptimal pH levels predominantly used in the cultu re medium. ii) Determine the various levels of 6-benzylaminopurine (BAP) and Kinetin (N6-furfu ryladenin) during the spread of the local Yemeni ginger variety. The findings could help to establis h and spread cultivation of ginger in different en vironmental conditions in Yemen by providing he althy and economic seedling.

Materials and Methods

The present experiment was conducted in Plant Tissue Culture Unit, Faculty of Agriculture-Sana'a University, Yemen during the period from January 2012 to June 2012 and aimed to establish a suitable and reproducible protocol for *in vitro* regeneration of local ginger (*Zingiber Officinale* Rosco).

Plant materials and source collection

A local variety of Yemeni ginger applied as a plan t substance in this experiment. It collected from Malhan highlands region located at west of Al-Ma hwit province (15°28 10 N 43°32 43 E) north-we st of capital city of Yemen (Fig. 1). Rhizomes pres erved in a mixture of peat moss and sandy soil in the laboratory to get growing shoots used as expl ants. Rhizome buds 1-2 cm long selected as initia l explants. Collected fresh buds cleaned and left u nder running water of the faucet for ½ - 1 h. Afte rwards, the buds were immersed and agitated in 20% (w/v) of Clorox (5.25% w/v of sodium hyp ochlorite) added with drops of Tween 20 with co nstant agitation. Next, they washed with sterile d istilled water for five times. Under aseptic conditi ons, bud scales peeled off and later trimmed to a bout 0.5 cm long. Medium complemented by vari ous hormones used according to treatments in c ulture medium for the regeneration of shoots an d create of roots from shoots multiplied for prod uct seedlings.

Fig. 1. Location of Al-Mahwit governorate (Red point) (located in northwest of capital city of Yemen (Sana'a). It is located at an elevation of about 2000 meters above the sea level. The climate of Al-Mawhit is considered as *Aw* (tropical savanna climate with dry-winter characteristics) according to the Köppen-Geiger climate classification. The average temperature in Al Mahwit is 23.1 °C. The rainfall is around 1189 mm per year. (https://en.climate-data.org/asia/yemen/al-mahwit-governorate/al-mahwit-54923/).

Experimental design to induce multiple shoots

Two experiments were performed in the tissue culture laboratory of the University of Sana'a:

The first experiment was done to find the most powerful pH medium. To do so, an experiment based on completely randomized block design was conducted with four replicates. The MS (Murashige and Skoog 1962) medium was used + 1 mg L^{-1} IBA at various pH levels (5.6, 5.8, 6.0). The right-most pH selected from this experiment was used in the second experiment.

In the second experiment, to test the best cytokinin type and level, a factorial experiment based on completely randomized block design was conducted with four replicates, including an experimental unit containing 10 test tubes. Two factors involved: i) Types of cytokinin: 6-benzyla minopurine (BAP) and Kiniten (Kin) (N6-furfuryladenine) and ii) Cytokinin concentrations (0, 1, 2 and 3 mg L^{-1}). The MS basal medium supplemented with above types and concentration of cytokinin with 1.0 mg L^{-1} IBA and 3% sucrose, the medium solidified with 0.7% agar at pH 5.8 and sterilized by using autoclaving for 8 min.

Culture condition

Properly sterilized sprouts were longitudinally cutting into 5-10 mm in length in the laminar airflow cabinet, and directly cultured on shoot regeneration medium containing 15 ml of MS medium supplemented with various concentrations of BAP and Kin as per treatments. One piece inoculated in each tube containing sterile culture medium with different concentrations of plant growth regulators for the shoot and root induction and then transferred to growth room and allowed to grow in a controlled environment. The temperature of the growth room maintained at 25±2 °C with an air conditioner, light period of 16 h maintained with an intensity of 2000 lux for growth and cultivation development.

Measurements

After 20 days of first experiment (pH levels), and after 6 weeks of second experiment (cytokinin

types and levels), the shoot length, shoot number and root number was recorded for all culture tube. Rooted shoots removed from the culture tu be and the root washed under running water to r emove the agar. For hardening, the seedlings tra nsferred to plastic pots containing peat moss and covered with polyethylene (Fig. 5A and B).

Data analysis

The data was statistically analyzed based on a RCBD, pH experiment was analysis as one way ANOVA with four replication, and the cytokinin experiment was analysis as a two-way ANOVA with four replicates using *Genstat* 12 software. The least significant difference (LSD) was used for the comparison of means amongst the treatments.

Results

Effect of pH levels

The effect of pH levels on the average growth of the shoot and roots is show in Table 1. Medium with pH at 5.8 produced significantly more shoot and root than the other pH levels. It was the highest with about 152% and 141% on shoots length, 267% and 308% on shoots number and 276% and 159% on the root number as compared to their characteristics in the lowest 5.6 and highest 6 pH levels respectively. Meanwhile, the lowest values labelled at 5.6 pH level (Table 1, Fig. 2). The explant values were green without verification under all pH levels.

Table. 1: Effect of different medium pH levels on shoot and root development of Yemeni local ginger variety from shoot-tip explants

pH Levels	Shoot length(cm)	No. Shoot	No. Root
5.6	5.0 [b]	1.5 [b]	6.5 [c]
5.8	7.6 [a]	4.0 [a]	18.0 [a]
6.0	5.4 [b]	1.3 [b]	11.3 [b]
LSD $_{0.05}$	**1.287**	**0.8619**	**1.389**

Means from four replicates for different medium pH levels (5.6, 5.8 and, 6.0) are presented. Different letters in the same column indicate significant differences ($p < 0.05$, Fisher's least significant difference test). All medium with different medium pH levels contain 1 mg L^{-1} IBA.

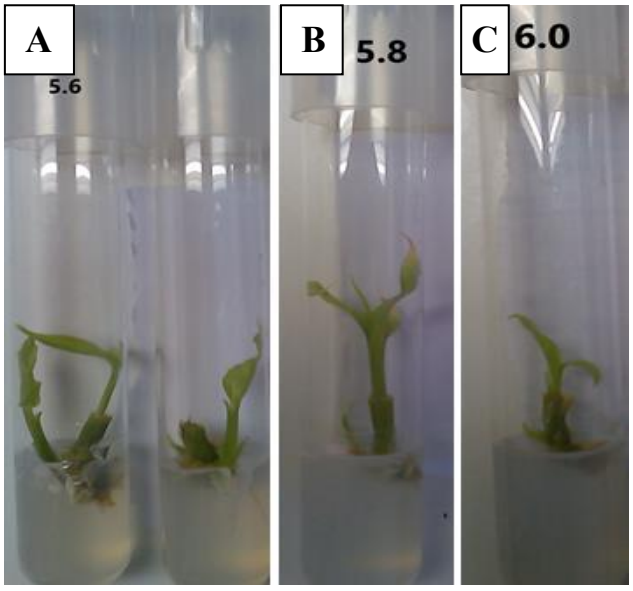

Fig 2: Effect of Different Levels of pH on Regeneration of Yemeni local ginger variety after 20 Days of culture. From the left: (A) : 5.6 , (B): 5.8 and (C) : 6.0

Effect of cytocinin type and concentration

In this study, cytokinin significantly affected the regeneration of local Yemeni ginger (Table. 2) and (Fig. 3 and 4). Overall, after 6 weeks of culture, Kin caused significantly the highest shoot number, shoot length, and root number by about 168.9%, 116.6%, and 173.6%, respectively, as contrasted with BAP.

Table 2. Effect of Cytokinin types (BAP and Kin) and its concentrations on the numbers of shoots and roots of Yemeni local ginger variety after 6 weeks of culture

**Treatments			No. Shoot	Shoot height (cm)	No. Root
Cytokinin (H)					
	BAP		1.875 [b]	4.75 [b]	9.12 [b]
	Kin		3.167 [a]	5.54 [a]	15.83 [a]
LSD $_{0.05}$			**0.3559**	**0.624**	**0.937**
Concentration (C) mg L^{-1}					
		0*	1.33 [c]	3.00 [a]	7.00 [a]
		1	3.25 [a]	6.50 [c]	14.42 [b]
		2	3.33 [a]	6.25 [c]	14.83 [b]
		3	2.17 [b]	4.83 [b]	13.67 [b]
LSD $_{0.05}$			**0.5033**	**0.882**	**1.325**
H*C					
	BAP	0	1.33 [c]	3.00 [c]	7.00 [d]
	BAP	1	3.50 [b]	6.33 [a]	11.83 [c]
	BAP	2	1.33 [c]	5.00 [b]	7.33 [d]
	BAP	3	1.33 [c]	4.67 [b]	10.33 [c]
	Kin	0	1.33 [c]	3.00 [c]	7.00 [d]
	Kin	1	3.00 [b]	6.67 [a]	17.00 [b]
	Kin	**2**	**5.33 [a]**	**7.50 [a]**	**22.33 [a]**
	Kin	3	3.00 [b]	5.00 [b]	17.00 [b]
LSD $_{0.05}$			**0.7117**	**1.247**	**1.873**

*Control (without cytokinin). All medium contain the cytokinin treatments +1 mg L^{-1} IBA, and pH is equal 5.8. Any two means in the same column for the same factor (Cytokinin types (H), Concentrations (C) or H*C) not followed by the same letter are significantly different ($p < 0.05$) using the analysis of variance and standard Fisher's protected LSD.

The data presented in Table 2 show the highest result of all parameters on MS medium supplemented with BAP or Kin at concentration of 2 mg L^{-1}. The higher or lower concentrations than 2 mg L^{-1} gave less significant results in all parameters with a similar concentration of 1 mg L^{-1}.

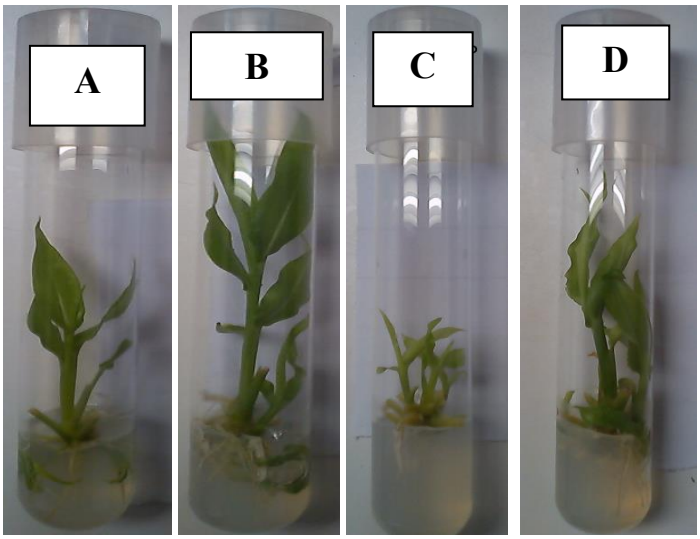

Fig. 3. *In vitro* micro-propagation of Yemeni local ginger variety, vegetative bud culture on MS fortified with different concentrations of BAP and multiple shooting and root induction after 6 weeks of culture. From the left (A) : 0.0 , (B): 1 mg L^{-1}, (C); 2 mg L^{-1}, (D): 3 mg L^{-1}

The significant difference between BAP and Kin is also clear in Table 2. One mg L^{-1} of BAP or Kin provides the same influence with no significant difference against shoots number and shoots height. Nevertheless, the number of the root was higher in one mg L^{-1} of Kin by about 43.7% than one mg L^{-1} BAP (Table 2). BAP at 1.0 mg L^{-1} proceeded in the highest multiple shoot length, shoot number and root number with about 210%, 262%, 168%, respectively, as compared with control treatment (with no hormones). There were no significant differences in on shoot number and shoot height at $p < 0.05$ between 1 and 2 mg/l of BAP.

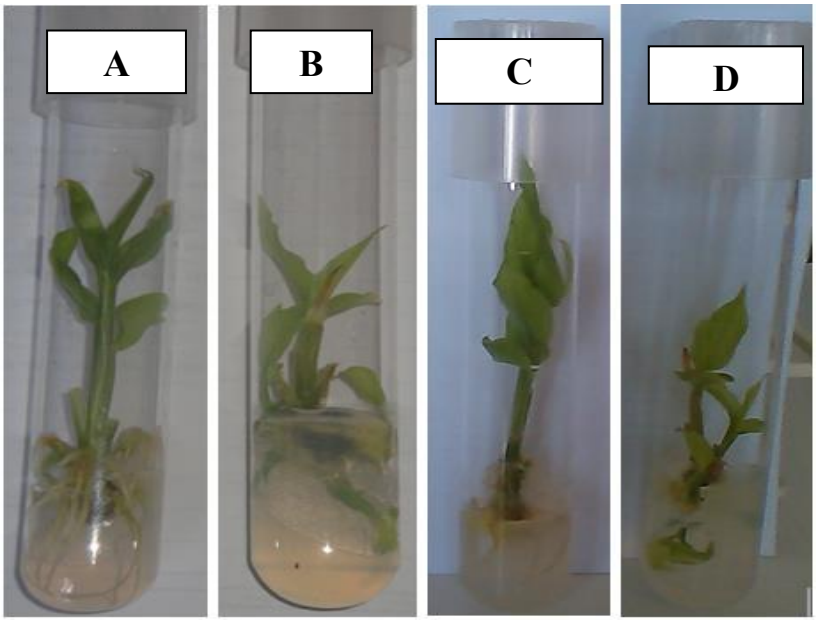

Fig. 4. *In vitro* micro-propagation of Yemeni local ginger variety, vegetative bud culture on MS fortified with different concentrations of Kin and multiple shooting and root induction after 6 weeks of culture. From the left: (A) : 0.0 mg L^{-1}, (B): 1 mg L^{-1}, (C); 2 mg L^{-1} and (D): 3 mg L^{-1}

The impact of Kin on the formation of the shoots and roots was clear, the concentration of 2.0 mg L^{-1} Kin produced the highest shoot number (5.3), shoot height (7.5) and root number (22.8) by about 400%, 250% and 319% higher compared to the control treatment, respectively.

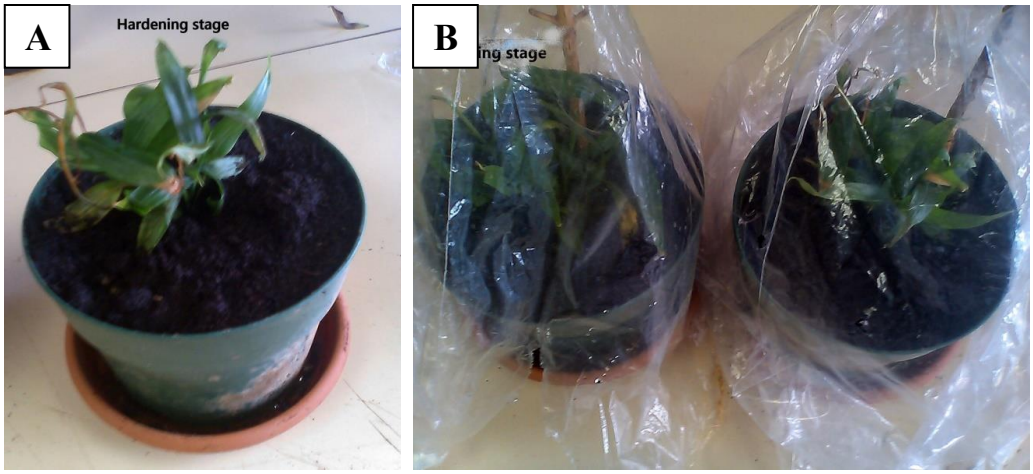

Fig. 5. Regenerated plant of Yemeni local ginger variety from sprout in plastic pot contain peat moss medium (A) and covered with polythene bag kept in lab for hardening (B).

Discussion

Micropropagation of plants *in vitro* mainly depen ds upon various factors and culture conditions. T he medium pH is also a critical factor among the m that correlated with plant growth under *in vitr o* conditions. The optimization of culture media p H is a necessary but critical step to achieve rapid and efficient growth of plantlets by using *in vitro* practices (Sharma et al.,2018). In the present stu dy, it was confirmed that the pH values play an i mportant role in the manipulation or manageme nt of the induction result of ginger explants. This finding is consistent with previous research cand idates by Rout and Das (1997) and Abdul Aziz et al. (2012).

Owen et al. (1991) stated that the pH of the medi a in tissue culture system could influence *in vitro* shoot multiplication, floral and secondary metab olites development, organogenesis, production of adventitious roots, and cell division. Whatever th e case, based on the previous studies, pH media was installed 5.8 as we consider it as the optimu m level in the current experiment like studies by Balachandran et al. (1990), Abbas et al. (2011), a nd Zahid et al. (2021).

As a matter of fact, plant growth regulators are o ne of the main factors affecting growth and devel opment of explant in tissue culture. Cytokinins pl ay major roles in shooting of different plants, eith er on foliage development *in vitro* (Rangan et al., 2004). Cytokinin stimulates cell division and incr eases the overall number of vegetative cells. It in crease growth rate, stimulates the rate of metabo lism of cells in the shoot, promotes storage of nut rients, increase active transport of nutrients and water, increase the flow of sugar and oxygen tow ard the site of syntheses (Rangan et al., 2004; Em ami et al., 2011; Al-madhagi 2012).

The positive effect of MS medium augmented wit h 2 mg L^{-1} Kin on increasing of shoots number, sh oot height and root number was take same direct ion with that found by Sharma and Singh (1997), who found the maximum number of shoots per b ud during culture initiation in the medium contai ning 2 mg L^{-1} Kin. Moreover, it is in accordance wi th Arildo et al. (2003) where Kin induces more s hoots by explant of *Tabernaemontana fuchsiaefol ia* L than the BAP. Also in agreement with Abu-Ro mman et al. (2015) who showed that Kin has mo st effective in inducing bud emergence from noda l explants of cucumber. Moreover, Hajare et al. (2 021) found the numbers of multiple shoots was differing between potato cultivars where, in one cultivar was the highest in the MS medium contai ning 2.5 mg L^{-1} Kin and other was when used PA B. Moreover, Buah et al. (2010) suggested that Ki n has the capacity to induce more banana shoots with increased concentrations at 7.5 mg L^{-1}. Hash emidehkordi et al. (2021) found that MS medium supplemented with 0.5 mg L^{-1} (IAA) + 0.1 mg L^{-1} Kin gave the highest rooting percentage and root number per explant of *Zantedeschia spp* and the highest root length was observed in 1.0 mg L^{-1} IA A + 0.1 mg L^{-1} Kin.

In contrast to our results, BAP was reported in m any studies such as by Balachandran et al. (1990), Abbas et al. (2011) and Zahid et al. (2021) as t he most effective cytokinin for shoot induction a nd multiplication in ginger. As well in other plant species such as *Daphne mezereum* L in the study by Nowakowska et al. (2019), who found that the highest number of shoots produced on MS medium with 1 mg L^{-1} BA and 0.1 mg L^{-1} NAA than the other types of cytokinins (meta-Topolin and zeatin).

The differences found in the relative strengths of the different cytokinin types in inducing shoots i n ginger are probably due to a mixture of factors such as stability, mobility, the rate of conjugation and oxidation of the hormones (Buah et al., 2010). This is also due to the antagonistic interaction between cytokinin and auxin in the MS medium (Kurepa et al., 2019). Where, the auxin/cytokinin ratio is the major physiological factor affecting shoot/root initiation (Rangan et al., 2004; Amin et al., 2007). It is clear that the cytokinin types have also major roles in this matter. Kurepa et al. (2019) concluded that *in vitro* auxin inhibits the response to low concentrations of cytokinin as a consequence of the BA, but is ineffective if the cytokinin content is high. The application of exogenous cytokinins *in vitro* resulted in an increase in the cytokinin content of the shoots, and application of zeatin and dihydrozeatin also detected in the newly-formed shoots. Application of BA to the media led to a transition from isoprenoid cytokinins to aromatic cytokinins in the shoots (Quiala et al., 2014).

This may clearly argues that the role of auxin on rooting, and increasing or decreasing endogenou s cytokinin may inhibit the role of auxin. Auxin and cytokinin play an important regulatory role in the plant, not only in apical dominance but also in many other correlated events as well and cytokinins exudates in the intact plants are under the control of the polar auxin transport system (Bangerth, 1994). Supporting to this role, Nirmal (1997) found that addition of NAA alone gives the highest root induction, whereas BAP alone at higher concentration (4 mg L^{-1}) induces only multiple shoots and rarely roots. Kambaska and Santilata (2009) found that the NAA is more effective than IBA in induction of ginger rooting as days required for rooting is lower by half of days as responsive to IBA. Adding 0.5 mg L^{-1} NAA

with BAP (2 mg L^{-1}) improve response over BAP alone and also it has been suggested that the combinations of BAP and NAA are needed for producing more number of multiple shoots on *Zingiber officinale* (Hoque et al., 1977 ; Hashim et al., 1998 ; Noguchi and Yamakawa., 1998).
In our experiment, the highest level of BAP and Kin (3 mg L^{-1}) gives the lowest result in all parameters with a similar concentration of 1 mg L^{-1}. These results are consistent with findings from Inden et al. (1988); Sharma and Singh (1997) and Anjumanara et al. (2003), who reported that high concentrations of cytokinin reduced shoot elongation, reduced rooting and caused genetic instability in micro-propagated ginger plants. Furthermore, Sharma and Singh (1997) indicated that low concentrations of Kin have a positive effect on *in vitro* shoot multiplication, where, plantlets obtained at lower concentrations of Kin had well-developed shoots, opened leaves and sparse and thin rooting, and at higher concentrations of Kin, less shoot elongation, folded leaves and very rare rooting were observed. This result also interpreted before by Teisson and Cote (1985), when over exposure to high level of cytokinins may lead to vitrification. In fact, the function of cytokinins or auxins is perspicuous on the concentration of other endogenous hormones in plants or in exogenous application (Rangan et al., 2004; Al-madhagi 2012).

Conclusion

It could be concluded that Kinetin is the best hormone for propagation of local Yemeni gainer with the concentration of 2 mg L^{-1}. Even though the role of cytokinin has been widely understood, the difference in the role of cytokinin types remains uncertain. It can be suggested that the effects of different types and concentrations of hormone should be analyzed on the level of endogenous hormone in tissue culture.

Acknowledgements

Authors are thankful with prayers and mercy to Dr. Taha Al-morish, the former head of tissue culture unit, Agriculture faculty, Sana'a University (who died of a stroke), for providing the Yemeni local ginger rhizome

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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