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Thidiazuron Role in *In vitro* Flowering of *Murraya paniculata* and *Fortunella hindsii via* Embryogenic Protoplast and Hypocotyl Segments Cultures

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ABSTRACT

Background: Procedures to regenerate protoplast into plants of citrus relatives is important to establish the genetic improvement for sweet oranges by using somatic hybridization and transgenic. Protoplasts are a good material to genetically manipulate and to become gene transfer. This study aims to characterize the regeneration sequence of *Fortunella hindsii* and *Murraya paniculata* protoplasts *via* somatic embryogenesis into flowering plants *in vitro*. This study also describes the sequence of flowering *in vitro* originated from hypocotyl segments of plantlets of two species.

Methods: Protoplast sources that were isolated from callus of *Fortunella hindsii* and *Murraya paniculata.* Embryogenic callus was put in Erlenmeyer and mixed together with 0.4% macerozyme, 0.2% cellulose and 0.1% driselase and pH was adjusted to 6.0. Two drops of purified protoplasts were cultured with media supplemented with 2.0% glucose and 0.0, 0.001, 0.01, 0.1 or 1.0. 10.0 mg/l thidiazuron (TDZ) and thicken with 0.9% gelrite.

Result: The media supplemented with 0.001 mg/l TDZ yielded the maximum plating efficiency, while 0.001 mg/l TDZ produced the highest percentage of shoot formation, approximately 80%. After being cultured on the same TDZ concentration for 14 days, the protoplasts that survived developed cell walls. *Fortunella hindsii* and *Murraya paniculata* underwent somatic embryogenesis to grow into plantlets. Thidiazuron has demonstrated efficacy in forming flower buds that grow normally. *Murraya paniculata* and *Fortunella hindsii* shoots that emerged from hypocotyl segmments flowered in vitro on half-strength MT media containing 0.001 to 0.01 mg/l TDZ and 2-3% sucrose after two months of culture and they eventually went on to flower.

Key words: *Fortunella-hindsii, In vitro* flowering, *Murraya-paniculata*, Plating-efficiency, Protoplast.

INTRODUCTION

Thidiazuron (TDZ) encourages flowers in low concentration and there could be increased plant metabolism and reserve plants. Thidiazuron may produce high quality bud floral and develop to normal flowers (Olah *et al.,* 2003). Thidiazuron with suitable concentration have been increase the axillary bud and are possible to increase frequency flowering *in vitro*. Addition of TDZ to the culture, triggered the adventitious buds to develop into early flowering *in vitro*. Thidiazuron also belongs to the group cytokinin synthetic as well as the benzylaminopurine. Thidiazuron have a role in stimulating endogenous cytokinin and they play a role as a cytokinin oxidase inhibitor that is an enzyme (Parveen and Shahzad, 2010).

Fortunella hindsii is one of citrus relatives that are potentially to use as an genetic resources for quality improvement and make the resistance traits of citrus, because the fruiting time is relatively short (Fu *et al*., 2003; Chen, 2012; Jumin and Nito, 1996a; Jumin and Nito, 1996b). As a citrus relative's member, they are used as a garnish while they also could be consumed as a food substitute to the citrus. *Murraya paniculata* potentially to be used as rooting sources for citrus resistance to nematodes, water flooding, saline soil and substitution a genetics improvement (Swingle and Reece, 1967; Sykes, 1988; Raveh, 2012; Jumin, 2013). Apparently the both varieties are related to citrus which are the easiest to use as sources as stem grafting and establish somatic hybridization with sweet orange (*Citrus sinensis*). *Citrus sinensis* is one kind of sweet orange that is in the highest demand in the market mainly in Indonesia and south east Asian countries.

Procedures to regenerate protoplast into plants of citrus relatives is important to establish the genetic improvement for sweet oranges by using somatic hybridization and transgenic plants. Protoplasts are a good material to genetically manipulate and to become gene transfer (Ahmed *et al,* 2021; Thakur *et al.,* 2022; Chen *et al*., 2023). Plants regenerated from seedlings take a long time to reach flowering. *Fortunella hindsii* as rooting to make the citrus become dwarfed for garnish purpose. Plant grafting in the

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citrus could be used as a way to shorten the early flowering plants. Shoot resulted from regenerated protoplasts as alternatively as stems to be used for grafting of citrus.

These technologies may be utilized as an additional method for producing wide hybridizations via protoplast fusion in order to improve the rootstock. This study aims to characterize the regeneration sequence of *Fortunella hindsii* and *Murraya paniculata* protoplasts via somatic embryogenesis into flowering plants *in vitro*. This study also describes the sequence of flowering *in vitro* originated from hypocotyl segment of plantlets of two species.

MATERIALS AND METHODS

Plant materials

Plant Materials using protoplasts and seedling hypocotyl segments. Protoplasts were isolated from embryogenic calluses of *Murraya paniculata* and *Fortunella hindsii*. Seedling hypocotyl segments internode of cultured seeds of both species. The experiments were conducted in Biotechnology and Genetic Universitas Islam Riau, Jl Kaharuddin Nasution 113 Pekanbaru 28284 Indonesia during 2020 to 2024.

Protoplast sources

Protoplast sources that were isolated from callus of *Fortunella hindsii* and *Murraya paniculata* by using (Jumin and Nito. 1996a,b,c) procedures. Formation callus emerged from plantlets in the culture after 3 mounts sub-culturing on MT media. Media prepared to induce embryogenic callus with 5% glucosa and addition of plant 0.01 mg/l kinetin, 600 mg/l malt extract and growing under 52.9 $\mu mol.m^{-2}.s^{-1}$ light intensity and a photoperiod of 16 h at 25°C.

Isolation of protoplasts

Embryogenic callus was put in Erlenmeyer and mixed together with 0.4% macerozyme, 0.2% cellulose and 0.1% driselase and pH was adjusted to 6.0. After overnight incubation on the gyratory shaker, the culturing of enzymes and callus were separated by reciprocal shaker at 25 rpm, for 5 min and 3 times.

Protoplast cultures

Two drops of purified protoplasts were cultured with media supplemented with 2.0% glucose and 0.0, 0.001, 0.01, 0.1, 1.0. or 10.0 mg/l thidiazuron (TDZ) and thickened with 0.9% gelrite. The culturing protoplasts were maintained for 40 days at 25°C temperature, 16 h photoperiod at 18.7 $\mu mol.m^2.s^{-1}$ light intensity. Furthermore, protoplasts developed into embryogenic callus and were maintained in the growth chamber at 25°C with 54 $\mu mol.m^2/s$ light intensity. Plating efficiency was counted as the percentage of living cells which become cells clustered in the media during 70 days following (Jumin and Nito, 1996c) procedures. The cell wall recoveries of the protoplasts was checked by fluorescein diacetate staining under an inverted microscope beginning from 10 days of culture.

Embryo induction and development

Sub-culturing of living cells has been subcultures 2 times every 30 days beginning from 30 days of living cell cultures till 90 days using media MT media supplemented with 2.0% glucose, 0.0, 0.001, 0.01, 0.1, 1.0 and 10.0 mg/l thidiazuron. (TDZ) solidified with 0.2% gelrite. After somatic embryos emerged, about 2 mm in length, they were carefully transferred into media consisting of 4% sucrose with 0.001 mg/l TDZ and 0.3% gelrite, 6.0 pH at 25°C maintained for 80 days.

In vitro flowering

Embryogenic callus developed into globular embryos and then grew into cotyledon embryo-like structures after about 50 days of culture. Torpedo somatic embryos like-structure grow into plantlets with two or more leaves and transferred into fresh media with the same treatments. The percentage of flowering was checked beginning from 15 days till 70 days of culturing in the media at room temperatures 25°C with 54 $\mu mol.m^{-2}s^{-1}$ light intensity and 16 days photoperiod.

Hypocotyl sources

Hypocotyl segments of cultured seedlings of *Murraya paniculata* and *Fortunella hindsii* were cut to 5 segments 5 mm in long and they were cultured into half-strength MT media consisting either 0.0, 0.001, 0.01, 0.1, 1.0 and 10.0 mg/l TDZ and either 2.0%, 3% and 4% sucrose and distributed to 25 × 150 mm glass tubes.

RESULTS AND DISCUSSION

Fresh protoplasts 30 minutes post isolation a diameter of 20-30 μm was cultured in half strength MT medium (Fig 3A). About 80% of the protoplasts were successful in recovering the cell wall and growth normally (Table 1, 2, Fig 3B) Cells grow into colonies 70 days after cultures and develop into globular somatic embryos (Table 3). Media with 0.001 mg/l TDZ significantly triggered cells to develop cotyledon somatic embryos-like structure and grow to torpedo shaped embryos like-structures about 50 days after

Table 1: Effect of TDZ and sucrose on plating efficiency (%) *Murraya paniculata*.

TDZ (mg/l)	Sucrose (%)		
	2.0	3.0	4.0
0.0	40.3a	38.6a	14.3a
0.001	80.3b	51.6b	15.6ab
0.01	74.6bc	40.3a	13.3a
0.1	64.3dc	19.6c	12.3a
1.0	44.6d	18.3c	11.6a
10.0	30.6e	15.6c	7.3b

According to Duncan's multiple range test, the mean value followed by a different alphabet column does not substantially differ from one another at P≤0.05.

cultures (Table 4). Torpedo shaped somatic embryos like structures grow into plantlets.

The relationship between shoot formation and flowering in vitro showed as interdependent in linear equation with y= 0.7542x+11.608 R^2 = 0.9029 (Fig 1) for *Fortunella hindsii* and y= 1.426x-12.541 R^2 = 0.9602 for *Murraya panicuata* (Fig 2). Meaning that an increase of shoot formation followed by an increase of flowering and they are connected to each other as a linear pattern. As a result there's an improvement in the somatic embryos and that is also an increasing percentage of flowering of the plantlets *Fortunella hindsii* and *Murraya paniculata* (Fig 4).

Murraya paniculata look more responsive to TDZ and sucrose than *Fortunella hindsii*. However both species have been sensitive to low concentration of TDZ. Concentration 0.001 mg/l TDZ is the best concentration to trigger protoplasts to recover the cell walls. The same response that the low concentration of TDZ is also significantly recovered cells grow into globular somatic embryos like-structure and to form cotyledon embryos like-structures. The concentration of 0.001 mg/l TDZ significantly triggered cells to develop cotyledon somatic embryos-like structure and grow to torpedo shaped embryos like-structures about 50 days after cotyledon somatic embryo like-structure cultures (Table 3). Torpedo embryos like-structures grow into plantlets (Ralker *et al*., 2008;, Neumann *et al.,* 2020).

Murashige and tucker media supplemented with cytokinin (TDZ) after the hypocotyl segments of *Fortunella hindsii* and *Murraya paniculata* grew to make the plantlets with more leaves. The number of leaves in each plantlet is averaged 2 to 4 leaves.

Media supplemented with cytokines supported the tissues to grow and become new tissues (Murashige and Tucker, 1969; Abubakar and Pudake, 2019; Fonseka and Aluthgamage, 2021).

At concentrations ranging from 0.001 to 0.10 mg/l TDZ, *in vitro* shoots of *Murraya paniculata* and *Fortunella hindsii* exhibited a favorable reaction to generate plantlets. With 80% of the hypocotyl segments developing into shoots and 75% beginning blossoms. TDZ at 0.01 mg/l shown to be the most effective to develope in flower *in vitro* (Fig 3C). A study by Srinivasan and Mullin (1978) indicated that endogenous cytokinin in ascending xylem sap is triggered by exogenous TDZ, hereafter stimulating flower buds and then causing the plant to initiate flower development.

Murraya paniculata protoplast and hypocotyl seedling near apical meristems using exogenous cytokinin to generate flowers *in vitro*. Moreover, *Fortunella hindsii* and *Murraya paniculata* hypocotyl segments initiated the mitotic cycle, which typically occurs prior to flowering. Bernier *et al.* (1977) discovered that cytokinin (TDZ) given to citrus relatives' protoplasts and plantlets may cause *in vitro* flowering. When *Fortunella hindsii* and *Murraya paniculata* plantlets generated from protoplasts developed in to flower bud in media supplemented with 0.001 mg/l TDZ and 2.0% sucrose and then flowering frequency rise. Media containing 2.0% sucrose and 0.00 mg/l TDZ added to media, the hypocotyl segment produced the best flower blooming (Fig 1, 2). According to Jumin and Ahmad (2000), when plantlets were cultured in MT media with 0.01 mg/l cytokinin (BA) and 5% sucrose, *Fortunella hindsii* and *Murraya paniculata*

Table 2: Effect of TDZ and sucrose on plating efficiency (%) *Fortunella hindsii.*

TDZ (mg/l)	Sucrose (%)		
	2.0	3.0	4.0
0.0	36a	48.3a	9.3ab
0.001	70.3b	52.3a	10.6a
0.01	64.6bc	50.6a	8.3ab
0.1	56.3dc	22.6b	7.3ab
1.0	46.6d	21.6b	5.6ab
10.0	20.6e	17.6b	4.3b

According to Duncan's multiple range test, the mean value followed by a different alphabet column does not substantially differ from one another at P≤0.05.

Table 3: Effect of TDZ and sucrose on development of protoplast-derived globular somatic embryos (%) *Fortunella hindsii* 40 days after culture (30 globular somatic embryos tested for each treatment).

TDZ (mg/l)	Sucrose (%)		
	2.0	3.0	4.0
0.0	60.3a	50.3a	39.0a
0.001	65.6a	60.3a	50.0ab
0.01	72.6a	63.6a	70.3c
0.1	62.3a	50.0a	60.3cb
1.0	40.3b	30.3b	50.6ab
10.0	27.6c	20.6c	11.3d

According to Duncan's multiple range test, the mean value followed by a different alphabet column does not substantially differ from one another at P≤0.05.

Table 4: Effect of TDZ and sucrose on shoot formation of *Murraya. paniculata* 50 days after torpedo shaped embryos like-structure culture (20 torpedo shaped embryos like-structure tested for each treatment).

TDZ (mg/l)	Sucrose (%)		
	2.0	3.0	4.0
0.0	48.6a	47.6a	37.0a
0.001	71.6a	41.3a	39.6b
0.01	67.3b	78.6b	76.3c
0.1	54.6b	79.3b	69.6c
1.0	41.6ab	67.3b	57.0bc
10.0	42.3c	40.6a	34.0d

According to Duncan's multiple range test, the mean value followed by a different alphabet column does not substantially differ from one another at P≤0.05.

floral buds grew into regular flowers. If there are high quantity of cytokinin, plantlets are sufficient to develop flower buds (Jumin and Ahmad, 2000; Scorza and Janick, 1980; Sing, 2023). Most flowers were normal and complex and appeared in the leaf axils. In Suitable concentration of cytokinin flowers fromed *in vitro* are common in citrus relatives when media, photoperiod, temperature, light intensity and other factors are supported (Jumin and Nito, 1996a,b; Jumin, 2016; Jayaraman and Ramachandran, 2024).

Murraya paniculata protoplast and hypocotyl seedling apical meristems using exogenous cytokinin to generate flowers *in vitro*. Moreover, *Fortunella hindsii* hypocotyl segment initiated the mitotic cycle, which typically occurs prior to flowering. Bernier *et al.* (1977), Jumin and Nito (1996a,b) discovered that cytokinin (TDZ) given to citrus relatives' protoplasts and plantlets may cause *in vitro* flowering. when plantlets were cultured in medium with 0.01 mg/l cytokinin (BA) and 5% sucrose, floral buds

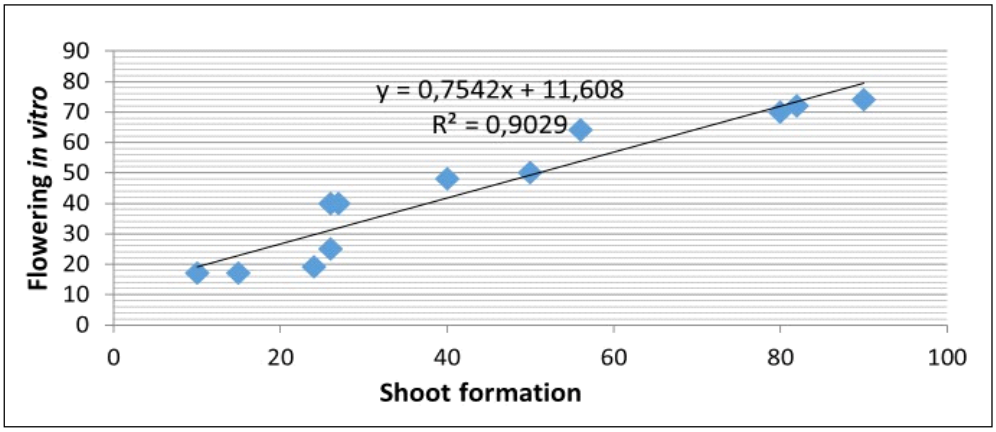


Fig 1: Relationship between shoot formation and flowering *in vitro* of *Fortunella hindsii.*

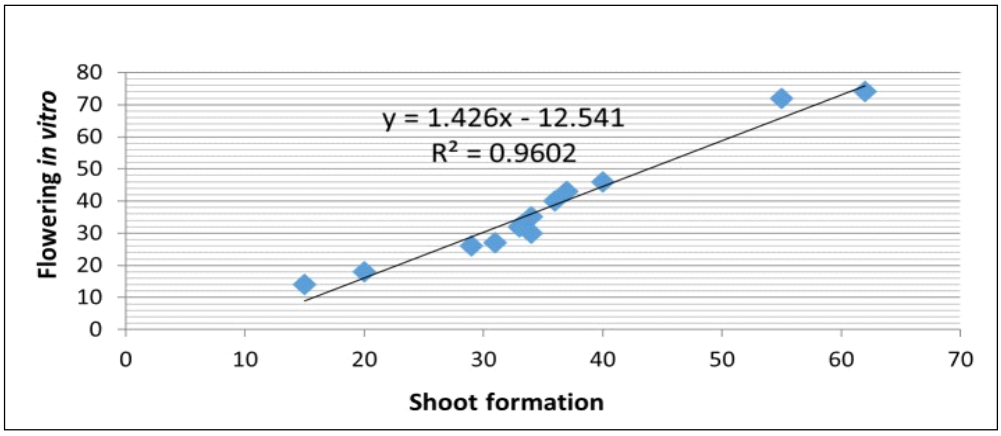
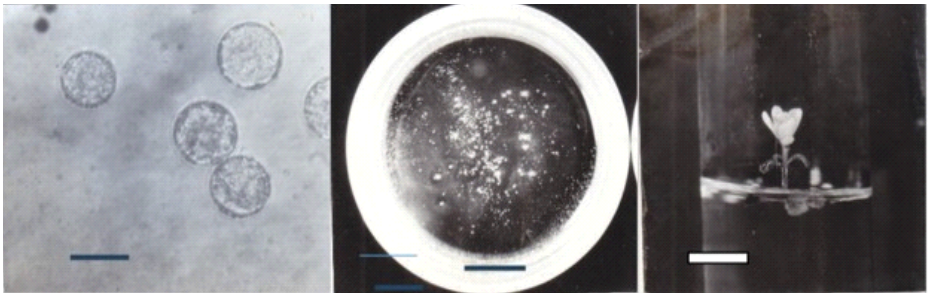


Fig 2: Relationship between shoot formation and flowering *in vitro* of *Murraya paniculata.*



(Center) Colony formation 75 days of culture (bar 5 mm), (Right). *In vitro* flowering of *Murraya paniculata* cultures to MT medium supplemented with 0.001 mg/l TDZ and 2.0 % sucrose (bar 1 cm).

Fig 3: (Left). Fresh protoplasts 30 minutes after isolation (bar 30 mm).

in *Fortunella hindsii* and *Murraya paniculata* grew into regular flowers.

Flower formation occurred when *Fortunella hindsii* and *Murraya paniculata* plantlets derived from protoplasts cultured in media supplemented with 0.001 mg/l TDZ and 2.0% sucrose. The optimal flowering originating from hypocotyl segments occurred from media with 0.00 mg/l TDZ and 2.0% sucrose. This is Jumin and Mukhtar (2000) mentioned that, In *Fortunella hindsii* and *Murraya paniculata* floral buds developed into complete and normal flowers when plantlets cultured in media at 0.01 mg/l cytokinin (BA), 5% sucrose. Plantlets are proper to form floral buds if 0.01 mg/l BA (cytokinin) and 2.0% sucrose mixed to media (Scorza and Janick, 1980; Jumin, 2016).

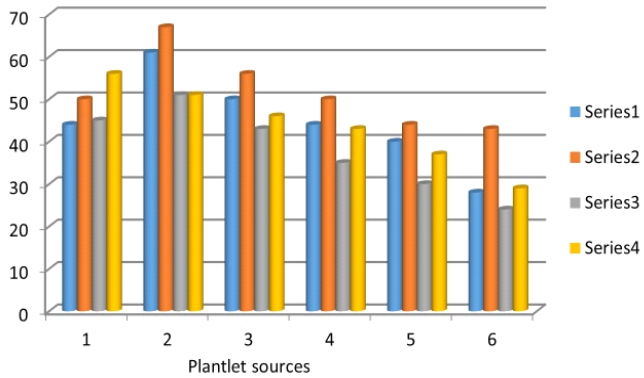
Flower formation gradually supple above 0.01 mg/l TDZ The optimal concentration of TDZ for flowering *in vitro* in both species has only 0.001 mg/l TDZ and 2.0% sucrose. The frequency of flowers declined gradually after 0.01 mg/l TDZ and 3.0% sucrose. With the optimal TDZ concentration and sucrose, there were a mean of 2 buds formed from the inflorescence developed from hypocotyl segments *Fortunella hindsii* and *Murraya paniculata* resulting from the floral buds from hypocotyl segments or plantlet derived from protoplasts. The tobacco thin cell layer stem originated from explants that produced either flower at suitable media, while leaves emerged at unsuitable media (Cousson *et al*., 1981).

Floral gradient for plantlets formation and flower *in vitro* on the first flower was observed between the hypocotyl segments positions if taken from the base of the hypocotyl segments during 80 days of culture. The plantlets derived from segments of hypocotyl segment commence flowers just if they were pieced node from stem originating close to the apex and cultured to media with proper plant hormone (Jumin, 2016; Cousson *et al*., 1981).

Plantlets originating from hypocotyl segments at the peak exhibited the highest percentage of flower development and as the distance from the peak increased, flowering emerged at a steadily decreasing rate (Fig 4). These findings suggest that there is a "floral gradient" in the position of hypocotyl segment both young and aged tissue. The largest percentage of in vitro blooms for *Murraya paniculata and Fortunella hindsii* is seen when the hypocotyl segment started near to the apex (Jumin and Nito, 1996; Jumin and Nito, 1996b).

They have the ability to produce flowers that descend basipetally from the hypocotyl segments base of hypocotyl. This relates to the finding that there is a gradient in the ability of flowers to regenerate, with the position of the shoots and flowers on the hypooctyl segments clearly influencing their production *in vitro*. According to Scorza (1982); Scorza and Janick (1980) and McDaniel *et al*. (1989) flower buds derived from hypocotyl segments developed into normal declining from the apical to the basal regions of hypocotyl segments. These findings suggested that endogenous gradients of growth substances in the stem may be the cause of this apex-to-base floral gradient.

The floral variation may result from changes in a cell's ability to produce or respond to a floral stimulus, from a gradient of a floral promoter or inhibitor at the time of explant excision, or from a gradient that combines promoter and inhibitor When trying to obtain de novo flowers in thin-cell-layer explants of a photoperiodically sensitive tobacco cultivar, the position of the branch internodes in the se on the intact plant is essential (Tran-Thanh-Van, 1973; Tran Thanh Van *et al.,* 1974; McDaniel *et al*., 1989; Wardell and Skoog, 1969; Lang, 1987); Bridgen and Veilleux, (1985); Rajeevan and Lang 1987) and Altamura *et al*. (1989) mentioned that because there is insufficient capacity to generate the flowering factor (s) or for the meristems to respond to the flowering factor, the position of the hypocotyl segments is insufficient to commence flowering (Lang, 1965; Hackett, 1985; Wardell and Skoog, 1969a). Tobacco branches have been shown to be able to generate flowers *in vitro* (Wardell and Skoog, 969a; Wardell and, Skoog, 1969b), while young *Passiflora suberose* leaf discs (Scorza, 1982)



(series 1) *Fortunella hindsii* Protoplasts, (series 2) *Murraya paniculata* protoplasts, (series 3) *Fortunella hindsii* hypocotyl segments, (series 4) *Murraya paniculata* hypocotyl segments).

Fig 4: Percentage of flowering *in vitro* on plantlet sources.

are unable to produce flowers. According to the current findings, a significant portion of branch internodes could generate blooms. Based on Chang and Hsing (1980) reported that mature *Panax ginseng* root callus was used to produce embryos, which in turn produced flowers with viable pollen. This effective system developed into a model that could be used to examine how feeding affects the reproductive activities of plants.

CONCLUSION

Surviving of isolated protoplasts in media supplemented by suitable concentration thidiazuron (0.001 mg/l) and sucrose (2.0%) grow into embryogenic callus, globular somatic embryos and then grow into spherical heart shaped somatic embryos-like structure, further then into plantlets. Plantlets originated from the branch internode of *Murraya paniculata* and *Fortunella hindsii* induced flowers *in vitro* under low concentration of thidiazuron and sucrose.

This efficient system from the protoplasts to somatic embryos and to plantlets in vitro of two species of have been potentially used as genetic manipulation to repair the genetics of these two species.

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Conflict of interest

All authors declare that they have no conflict of interest.

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