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Enhancement of shoot proliferation and evaluation of biotic elicitation effects on anatomical changes of pseudostem and anti-lipid peroxidation activity of *Curcuma mangga* Val.

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Enhancement of shoot proliferation and evaluation of biotic elicitation effects on anatomical changes of pseudo stem and anti-lipid peroxidation activity of *Curcuma mangga* Val.

Abstract: Mango turmeric (*Curcuma mangga* Val.) contains many bioactive compounds that are used for traditional treatment of various health problems and ailments. Slow propagation nature of *C. mangga* have resulted in short supply to meet the market demand. The longitudinally incised half shoot explants promote 100 % increased of shoot number compared with non-incised shoots with the formation of average 6.6 shoots/explant when they were cultured either vertically or horizontally on MS medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA. Biotic elicitation with 3.5 mg l^{-1} or 5.0 mg l^{-1} yeast extract or combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract did not promote shoot proliferation but exhibited anti-lipid peroxidation activity slightly lower than quercetin, a potent plant antioxidant flavonoid and butyl hydroxyl toluene (BHT), a commercial preservative agent which is used as a positive control. While absolute ethanol which served as a negative control did not show any anti-lipid peroxidation activity. Biotic elicitation of *C. mangga* plantlets using similar elicitors resulted in anatomical changes of its pseudostem with reduced number of thin lignified xylem cells and the presence of druse suspected to be oxalate crystals inside the cortex cells with delicate cell wall.

Key words: anti-lipid peroxidation activity; chitosan; mango turmeric; pseudostem; shoot proliferation; yeast extract

Pospeševanje tvorbe poganjkov in ovrednotenje elicitacijskih učinkov na anatomske spremembe navideznih stebel in proti maščobne peroksidacijske aktivnosti kurkume (*Curcuma mangga* Val.)

Izvleček: Kurkuma (*Curcuma mangga* Val.) vsebuje številne bioaktivne snovi, ki se uporabljajo pri tradicionalnem obravnavanju številnih zdravstvenih težav in obolenj. Počasen način njenega razmnoževanja povzroča njeno pomanjkanje glede na veliko povpraševanje na trgu. Do polovice vzdolžno zarezani stebelni izsečki so stoodstotno povečali število poganjkov v primerjavi z nezarezanimi s tvorbo povprečno 6,6 poganjkov na izseček, če so bili gojeni navpično ali vodoravno v MS gojišču, obogatenim z 2,0 mg l^{-1} BA in 0,5 mg l^{-1} NAA. Biotično vzpodbujanje s 3,5 mg l^{-1} ali 5,0 mg l^{-1} izvlečka kvasa v kombinaciji z 150 mg l^{-1} hitozana in 3,5 mg l^{-1} izvlečka kvasa ni pospešilo tvorbe poganjkov ampak je pokazalo malo manjšo antiperoksidacijsko aktivnost v primerjavi s kvercetinom, močnim rastlinskim flavonoidnim antioksidatom in butil hidroksi toluenom (BHT), komercialnim zaščitnim sredstvom, ki sta bila uporabljena kot pozitivna kontrola. Uporaba absolutnega etanola kot negativne kontrole ni pokazala nobene antiperoksidacijske aktivnosti. Biotično vzpodbujanje nastanka rastlinic korkume s podobnimi eliciatorji je povzročilo anatomske spremembe v nastajajočih navideznih steblih z zmanjšanjem števila tankih lignificiranih celic ksilema in prisotnostjo kritalnih kopuč, domnevno iz kalcijevega oksalata, v celicah primarne skorje, ki so imele zelo tanko celično steno.

Ključne besede: antiperoksidacijska aktivnost za maščobe; hitozan; kurkuma; navidezna stebla; tvorba poganjkov; izvlečki kvasa

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1 INTRODUCTION

Mango turmeric (*Curcuma mangga* Val.), a member of Zingiberaceae family, is commonly used in Ayurvedic, traditional Chinese medicine (TCM) and alternative Malay medicines (Ramadanil et al., 2019). It has been used as food spices, food preservatives and treatment of various health problems and minor ailments such as fever, stomach-ache, general debility, and postpartum care (Subositi and Wahyono, 2019; Furmuly and Azemi, 2020). It has been proven to have many health beneficial activities due to some bioactive compounds or secondary metabolites such as the alkaloids, flavonoids, tannins, terpenoid and curcuminoid that are present mainly in the rhizomes (Muchtaromah et al., 2018; Yuandani et al., 2019; Awin et al., 2020; Fitriastuti et al., 2020; Maryam and Martiningsih, 2021).

Conventionally, *C. mangga* is propagated via its rhizome during the rainy season. However, *C. mangga* and many other *Curcuma* species have slow propagation rate and very prone to soil borne diseases and rhizome dormancy (Škorničková, 2007; Pikulthong et al., 2016; Soonthornkalump et al., 2020; Leong-Škorničková et al. 2021). This has resulted in insufficient supply of *C. mangga* to meet the market demand. *In vitro* culture techniques have been employed as alternative measures for improving the production of plantlets for some *Curcuma* species such as *C. longa* (El-Hawaz et al., 2015; Marchant et al., 2021), *C. aromatica* (Sharmin et al., 2013; Mohanty et al., 2015), *C. alismatifolia* (Li et al., 2021) and *C. zedoaria* (Sudipta et al., 2020). Addition of elicitors into culture medium had been reported to enhance the production of useful secondary metabolites but high amount of elicitation was found to cause cell or tissue damage of the cultured materials (Espinosa-Leal et al., 2018). Our previous study (Abraham et al., 2011) had reported that biotic elicitation using yeast extract and chitosan did not enhance shoot proliferation but increased the production of total phenolic compounds which resulted in severe abnormality of the *C. mangga in vitro* plantlets especially the pseudo stems. Hence, the present study was carried out with three main objectives. Firstly, to determine whether a shoot incision technique could enhance *in vitro* shoot proliferation and used it as an alternative means for mass production of *C. mangga* plantlets. Secondly, to determine whether yeast extract and chitosan that have been found to promote production of total phenolic compounds with good free radical scavenging activity could also enhance the anti-lipid peroxidation activity of *C. mangga*. Thirdly, to study the effect of biotic elicitation of yeast extract and chitosan on the anatomical structures of *C. mangga* pseudostem.

2 MATERIALS AND METHODS

2.1 ESTABLISHMENT OF *IN VITRO* PLANTLETS

Young buds of *C. mangga* of approximately 1.5 cm^3 in size, excised from the actively growing rhizomes during the raining season. They were washed with commercial detergent solution (Sunlight®, Unilever, Selangor, Malaysia) to remove all soil and organic matters and rinsed under running tap water for 40 min. The cleansed bud explants (1.5 cm^3) were then immersed in 70 % ethanol (Chemical Industries (M) Sdn. Bhd., Selangor, Malaysia) for 10 min followed by surface sterilization with 20 % Clorox®, a commercial bleach solution containing 5.3 % sodium hypochlorite (The Clorox Company, Oakland, CA), for 20 min. The surface-sterilized buds were then rinsed three times with sterile distilled water before being inoculated onto gelled MS (Murashige and Skoog, 1962) medium without any plant growth regulator (PGR). The cultures were incubated in a culture room regulated at 25 ± 2 °C with continuous illumination at average light intensity of 32.5 µmol m^{-2} s^{-1} for 6 weeks (Abraham, 2010).

2.2 EFFECT OF SHOOT INCISION ON SHOOT PROLIFERATION

The shoot explants of 1.0 cm length were obtained from the 6 weeks old *in vitro* plantlets. Each shoot explant was vertically cut into half or quarter while the non-incised whole shoot explants were used as control. These shoot explants were then cultured onto shoot proliferation medium, MS supplemented with 2 mg l^{-1} 6-benzylaminopurine (BAP) and 0.5 mg l^{-1} 1-naphthalene acetic acid (NAA) (Sigma-Aldrich (M) Sdn. Bhd. Subang Jaya, Malaysia) (Abraham, 2010). Three shoot explants were used for each experimental unit and ten experimental units were used for each explant type. The explants were placed vertically and horizontally on the proliferation medium. The number of shoots produced from each explant with different mode of placement was determined after 6 weeks of culture.

2.3 ANTI-LIPID PEROXIDATION ACTIVITY OF *CURCUMA MANGGA*

2.3.1 Sample extraction

Our previous study (Abraham et al., 2011) have indicated that plantlets cultured in proliferation medium added with 3.5 mg l^{-1} yeast extract, 5.0 mg l^{-1} yeast

extract, and plantlets cultured in proliferation medium supplemented with150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract produced high total phenolic compounds with high free radical scavenging activity (RSA). Hence, these plantlets together with plantlets cultured in shoot proliferation medium (Control) were selected for their anti-lipid peroxidation activity. Two grams (g) of dried sample derived from each treatment condition was grounded into powder form using blender (Philips, Selangor, Malaysia). Each sample was placed into 250 ml conical flask and soaked with 100 ml methanol (Chemical Industries Sdn. Bhd., Selangor, Malaysia) at 40 °C for two hours and the soaking process was repeated three times. The methanol extracts from each sample were collected, combined, filtered, and evaporated using rotary evaporator machine (EYELA, N-N Series, Japan).

2.3.2 Anti-lipid peroxidation activity

Anti-lipid peroxidation activity was determined using modified Ferric thiocyannate (FTC) method (Osawa and Namiki, 1981). *C. mangga* sample extract (4 mg) was dissolved in 4 ml absolute ethanol (99.5 %) followed by addition of 8 ml 0.05M phosphate buffer (pH 7.0), 4.1 ml 2.5 % linoleic acid solution (Sigma, Ronkonkoma, NY) and 3.9 ml distilled water. The extract solutions were kept in aluminium foil wrapped vessels and incubated at 40 °C. Butyl hydroxyl toluene (BTH) (Sigma, Ronkonkoma, NY) was used as a positive control and 4 ml absolute ethanol served as a negative control. A volume of 0.1 ml extract solution was added to 9.7 ml 7.5 % ethanol followed by 0.1 ml 30 % ammonium thiocyannate solution and 0.1 ml 0.02 M ferric chloride (Sigma, Ronkonkoma, NY) in 3.5 % HCl (v/v). The reaction was incubated for three minutes under dark condition. Three replicates were prepared for each sample and three repetition of spectrophotometer reading were applied for each sample. The absorbance of sample was measured at 500 nm wavelength using UV-Vis spectrophotometer (Mettler Toledo, Columbus, Ohio). This procedure was repeated every 24 hours until both the positive and negative controls gave the maximum absorbance. The degree of lipid peroxidation was represented by percentage of oxidized lipid in tested samples at the day before the absorbance decreased.

2.4 HISTOLOGY STUDY OF BASAL PSEUDOSTEM

The basal pseudostems of the morphological abnormal *C. mangga* plantlets were selected for anatomical study. They were trimmed into approximately 0.5 cm^3 in size and were then immersed in FAA solution (40 % formaldehyde: acetic acid glacial: 95 % ethanol = 5: 5: 90) for fixation. During preparation of sections, the fixed tissues were passed through a series of alcohol solutions, starting with 50 % ethanol/tetra butyl alcohol (TBA) (ethanol 95 %: absolute TBA: water = 4: 1: 5) and finally treated with absolute ethanol/TBA (ethanol 95 %: absolute TBA = 2.5: 7.5). After which the tissues were immersed in mixture solution of TBA and liquid wax with ratio 1:1 at 60-62 °C for 24 hours. At the embedding stage, the tissues were immersed in liquid wax at 60-62 °C for 12 hours. After the wax solidified, the wax blocks containing the tissue samples were sliced into thin slices with 15 µm thickness using rotary microtome (Leica, Germany) for slide preparation. Double stained standard technique was used for the preparation of permanent slides. Each sliced section treated with a few drops of safranin after it was placed on the glass slide. This was followed by a few drops of 95 % ethanol to remove the excess safranin. After this, a few drops of Fast Green and 95 % ethanol were added respectively. Finally, one drop of xylene was added on the section. The sliced section was then covered with a glass cover slip and sealed with Shandon Mount (Shandon, USA) as a mounting agent. The prepared slides were observed under light microscope (Olympus BX-50, Japan) fitted with coloured video camera (JVC KF-55B, Japan) and image analysing system (analySIS docu version 3.1, Germany) for determination of cell size and cell morphology.

2.5 STATISTICAL ANALYSIS

For the effect of shoot incision and mode of placement on shoot proliferation, the experiment was conducted in complete randomized block design (CRBD). The data was analysed using two-way ANOVA and the best explant type was determined using Tukey's HSD test at $p \leq 0.05$. The anti-lipid peroxidation activity for all the selected samples was analysed using one-way ANOVA and the comparison of means was determined using to Tukey's HSD test at $p \leq 0.05$

3 RESULTS AND DISCUSSION

3.1 EFFECTS OF SHOOT INCISION ON SHOOT PROLIFERATION

Shoot proliferation is an essential step for mass production of *in vitro* plantlets which is normally car-

ried out by inducing multiple shoots formation using plant growth regulators. Many researchers have been using 6-benzylaminopurine (BAP) or benzyl adenine (BA) combination with naphthalene acetic acid (NAA) for multiple shoot induction in *Zingiber* species (Abbas et al., 2011; Zahid et al., 2021) and *Curcuma* species (Bejoy et al., 2012; Jala, 2012; Ferrari et al., 2016) with the formation of an average of 3 to 5 shoots per explants. Similar result was obtained in the present study whereby an average of 3.3 and 3.7 shoots per shoot explant were formed when the shoot explants were cultured horizontally and vertically respectively on MS medium supplemented with 2.0 mg l^{-1} BA and 0.5 mg l^{-1} NAA for induction of multiple shoot formation. When the shoot explants were cut longitudinally into half, the number of shoots formed per shoot explant was greatly enhanced with the formation of 6.6 shoots per shoot explant and the number of shots formed were not significantly different when the explants were placed horizontally or vertically. The quarterly cut shoot explants further enhanced the formation of multiple shoots with 7.6 and 8.4 shoots per shoot explant formed when the shoot explants were cultured horizontally and vertically respectively (Table 1). However, the multiple shoots derived from the quarterly cut shoot explants were small and became necrotic with copious release of phenolic compounds in the culture medium and eventually resulted in death of plantlets after two subculture cycles (6 weeks/cycle). Mode of inoculation either vertically or horizontally was statistically found to have no effect on promoting shoot proliferation in *C. mangga*. The half shoot explants grow into healthy multiple shoots when cultured on the shoot proliferation medium. Hence, half shoot explants were used for the subsequent studies.

Results obtained in the present study clearly demonstrated that shoot incision longitudinally could enhance 100 % increment of shoot production. The enhancement of shoot proliferation in longitudinally incised shoots was due to increased cut surface area that exposed to the culture medium for better absorption of nutrients and inhibit apical dominance and hence induce more lateral shoot formation (Mok and Ho, 2019). Longitudinally dissecting of shoot explants to promote high multiple shoot formation has become a common practice in propagation of banana (Ahmed Hasan et al., 2020). Hence, shoot incision can be used as an alternative means to promote *in vitro* shoot multiplication of *C. mangga* that resulted in more and faster production of plantlets.

3.2 ANTI-LIPID PEROXIDATION ACTIVITY OF BIOTIC ELICITED *CURCUMA MANGGA*

Our previous study (Abraham et al., 2011) had shown that *C. mangga* plantlets, cultured in proliferation medium supplemented with 3.5 mg l^{-1} yeast extract; 5.0 mg l^{-1} yeast extract or combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract, exhibited high free radical scavenging activity (RSA). These crude extracts together with the positive and negative controls were tested for their anti-lipid peroxidation activity using the ferric thiocyannate (FTC) assay. This method was used to measure the antioxidant activity of the studied samples toward auto peroxidation of the linoleic acid. BHT (Butyl Hydroxyl Toluene) (Sigma, USA) was used as a positive control and 99.5 % ethanol served as a negative control. Positive control is essential for comparing the biotic elicited *C. mangga* extracts with BHT, a most used antioxidant as preservative in foods containing fats, pharmaceuticals, petroleum products and it inhibits autoxidation of unsaturated organic compounds. The *C. mannga* extracts used in this study were dissolved in 99.5 % ethanol, hence 99.5 % ethanol was used as the negative control. The negative control is used to show that any positive effects of the tested samples are not due to the ethanol effect. The selected *C. mangga* extracts together with BHT (positive control) and 99.5 % ethanol (negative control) and quercetin, a potent plant antioxidants flavonoid, exhibited similar pattern of lipid peroxidation activity from day 0 to day

Table 1: Effect of shoot explant incision and inoculation mode on enhancing multiple shoot formation of *C. mangga*

Explants	Vertical		Horizontal	
	No. of shoots/explant ± *Se*	No. of shoots/shoot	No. of shoots/explant ± *Se*	No. of shoots/shoot
Whole	3.7 ± 0.2	$3.7^{c}{}_{c}$	3.3 ± 0.3	$3.3^{c}{}_{c}$
Half	3.3 ± 0.3	$6.6^{b}{}_{b}$	3.3 ± 0.3	$6.6^{b}{}_{b}$
Quarter	1.9 ± 0.2	$7.6^{a}{}_{a}$	2.1 ± 0.2	$8.4^{a}{}_{a}$

Mean values within the row followed by same superscript letter indicate not significantly different when the different explant types were vertically or horizontally placed on the culture medium. Mean values within the same column (for parameter No. of shoots/shoot explant) followed by different subscript alphabet indicate significantlydifferent of shoot numbers for different explant types (Tukey, HSD, $p \leq 0.05$)

six. However, the oxidation of lipid (linoleic acid) of 99.5 % ethanol (negative control) drastically increased started from day seven until day ten while others remain constant until day 10 (Figure 1).

Quercetin is a potent antioxidant flavonoid found in many plant species such as onions, grapes, berries, broccoli, and citrus (David et al., 2016). Even though the three extracts obtained from biotic elicited plantlets of *C. mangga* showed slightly lower anti-lipid peroxidation activity when compared with quercetin, it could be assumed that *C. mangga* could also a potent antioxidant. Their anti-lipid peroxidation activity was also found to be lower when compared to BHT, a commercial preservative agent and a synthetic antioxidant. BHT and quercetin showed similar lipid-peroxidation activity with percentage of oxidized lipid as 29.5 ± 0.1 % and 30.0 ± 0.1 % respectively. Crude extract derived from *C. mangga in vitro* plantlets cultured in proliferation medium supplemented with 3.5 or 5.0 mg l^{-1} yeast extract exhibited no difference in anti-lipid peroxidation activity. Plantlets cultured in proliferation medium supplemented with 3.5 mg l^{-1} yeast extract showed better anti-lipid peroxidation activity as compared to extract derived from *C. mangga* plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract. Plantlets cultured in proliferation medium without elicitation exhibit the least inhibition of lipid peroxidation (Table 2). It opened an interesting possibility to use *C. mangga* extracts as food preservative agents or topical medication for treatment of cold sore such as BHT. Besides using as food preservative agent, BHT has been used as medicine for the treatment of cold sore (Freeman et al., 1985).

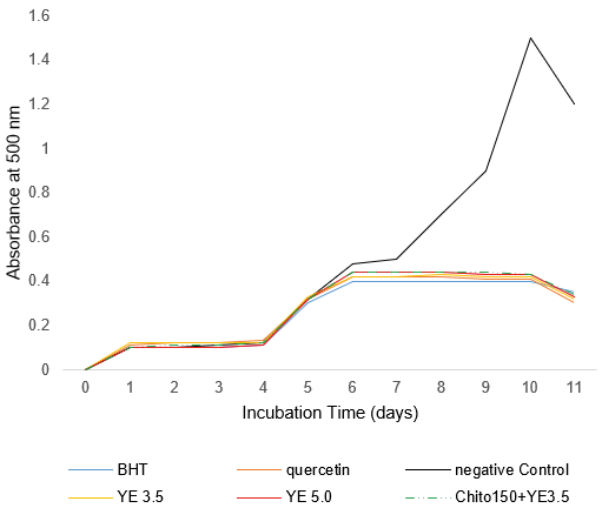

Figure 1: The kinetic of anti-lipid peroxidation of biotic elicited *C. mangga* extracts and controls [BHT (positive control); 95.5 % ethanol (negative control)]

3.3 EFFECT ON ANATOMY OF PSEUDOSTEM

Our previous study (Abraham et al., 2011) had shown that the morphology of the plantlets cultured in the proliferation medium without elicitation (Control) were normal. While those cultured in proliferation medium with the addition of yeast extracts (3.5 mg l^{-1} or 5.0 mg l^{-1} were slightly abnormal with retarded growth. Those plantlets cultured in proliferation medium with combination of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were grossly abnormal with chlorosis and globular shaped shoots, fragile leaf petiole and experience severe growth retardation. Results obtained from the histological study of the pseudostem in the present study (Figure 2, Figure 3 & Table 3) clearly showed that these elicited plantlets did affect the anatomical structures of the basal pseudostem of *C. mangga* and could be linked directly with the morphological characteristics of *C. mangga* plantlets as reported in Abraham et al. (2011). The plantlets cultured in the shoot proliferation medium without elicitation (Control) were consisted of smooth spherical to oval shape cortex cells

Table 2: Percentage of oxidized lipid in tested samples at day 10 using ferric thiocyanate (FTC) assay

Sample	Oxidized lipid in sample (%) ± se
Absolute ethanol (Negative Control)	100 a
Quercetine	30.0 ± 0.1 e
BHT (Butyl Hydroxyl Toluene) (positive control)	29.5 ± 0.1 e
Extract from plantlets cultured without biotic elicitors	33.0 ± 0.2 b
Extract of 3.5 mg l^{-1} yeast-extract treated plantlets	31.6 ± 0.2 d
Extract of 5.0 mg l^{-1} yeast-extract treated plantlets	32.1 ± 0.1 cd
Extract of 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast-extract treated plantlets	32.5 ± 0.1 c

Mean values within the column followed by same alphabets represent non-significantly different mean values based on Tukey, HSD at $p \leq 0.05$

and normal xylem cells. The cortex cells did not contain any druse, a crystal substance present in plant such as calcium oxalate crystal. The well distributed cortex cells without druse and well-formed lignified xylems (Figure 2 a & b) were able to supply sufficient nutrients to support healthy and normal plantlets. For the plantlets cultured in medium supplemented with 3.5 mg l^{-1} yeast extract, the xylems were also well-formed except the lignified layer of these xylem cells were not as thick as the ones observed in the control. Their cortex cells were found to contain druse, which could be seen as black dots inside the cells (Figure 2 c & d). The accumulation of druses was more obvious in plantlets that were cultured in proliferation medium supplemented with 5.0 mg l^{-1} yeast extract (Figure 2 e & f). The size of xylem cells of the control plantlets and that cultured in proliferation added with 3.5 mg l^{-1} yeast extract was not significantly different, with an average diameter of 32.9 ± 4.0 µm and 40.8 ± 5.1 µm respectively. They were double the size of the xylem cells present in the basal pseudostem of plantlets cultured in proliferation medium supplemented with 5.0 mg l-1 yeast extract (15.4 ± 0.8 µm). However, the number of xylem cells found in plantlets cultured with the presence of 3.5 mg l^{-1} yeast extract was only 22 when compared to that of control with 41 xylem cells. The plantlets cultured in medium supplemented with 5.0 mg l^{-1} yeast extract produced even much lesser number of not well-lignified xylems, with an average of only 9 xylem cells in each 4x optical magnification field (Table 3). The control plantlets developed bigger cortex cells (43.0 ± 3.0 µm) compared to plantlets cultured with the proliferation medium supplemented with 3.5 mg l^{-1} and 5.0 mg l^{-1} yeast extract with diameter of cortex cells as 29.9 ± 1.8 µm and 30.8 ± 1.8 µm respectively, which were not significantly different in size (Table 3). The smaller size of cortex cells with the presence of druse, and a smaller number of poor lignified xylem cells might not be able to provide sufficient nutrients derived from the culture medium to all parts of plantlets. Hence, it explained the retarded growth with abnormal characteristic of the plantlets that were cultured in proliferation medium supplemented with 3.5 mg l^{-1} and 5.0 mg l^{-1} yeast extract as reported in our previous study.

Most of the tissues of the plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were fragile, and parts of the section were damaged after dehydration process (Figure 3 a & b). The size of some of the cortex could be measured and determined but not that of xylem cells because of cell damage. The number of the xylem cells (7 in each 4x optical magnification field) was roughly estimated from the location of the ruptured xylem cells. The size of the cortex cells found in the basal pseudostem of *C. mangga* plantlets cultured in the shoot proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract was found to be not significantly different with those plantlets cultured in proliferation medium added with yeast extract (Table 3). The small number and fragile xylem cells could greatly reduce the absorption of nutrients, and this was linked directly with the severe growth retardation and gross morphological abnormality of the plantlets cultured in proliferation medium supplemented with yeast extract and chitosan as reported in Abraham et al. (2011). Yeast extract was reported to induce a complex stress response resulted in activation of secondary metabolites production (Farjaminezhad and Garoosi, 2021; Kochan et al., 2017) but resulted in slow cell growth (Hedayati et al., 2021) and cell damage at high concentration (Sánchez-Sampedro et al., 2005).

In the present study, the formation of druse (crystal inclusion inside cortex cells) was detected in *C. mangga* plantlets cultured in proliferation medium elicited with yeast extract. The druse inside the cells of *C. mangga* plantlets were suspected to be oxalate crystal because oxalate crystals are the most common crystal inclusion of higher plants (Franceschi and Horner,

Table 3: Summary of cortex and xylem diameter and estimated number of xylem cells in *Curcuma mangga* plantlets cultured in proliferation medium supplemented with biotic elicitors

Elicitor treated peudostems	Diameter ± s.e. (µm)		Estimated No. of xylems/ 4x optical magnification field
	Cortex	Xylem	
Control	43.0 ± 3.0 a	32.9 ± 4.0 a	41
5.0 mg l^{-1} yeast extract	30.8 ± 1.8 b	15.4 ± 0.8 b	9
3.5 mg l^{-1} yeast extract	29.9 ± 1.8 b	40.8 ± 5.1 a	22
150 mg l^{-1} chitosan + 3.5 mg l^{-1} yeast extract	35.2 ± 3.9 b	ND	7

Mean values within the same column followed by different alphabet indicate significantly different values (Tukey, HSD, $p \leq 0.05$).
ND = Not determine

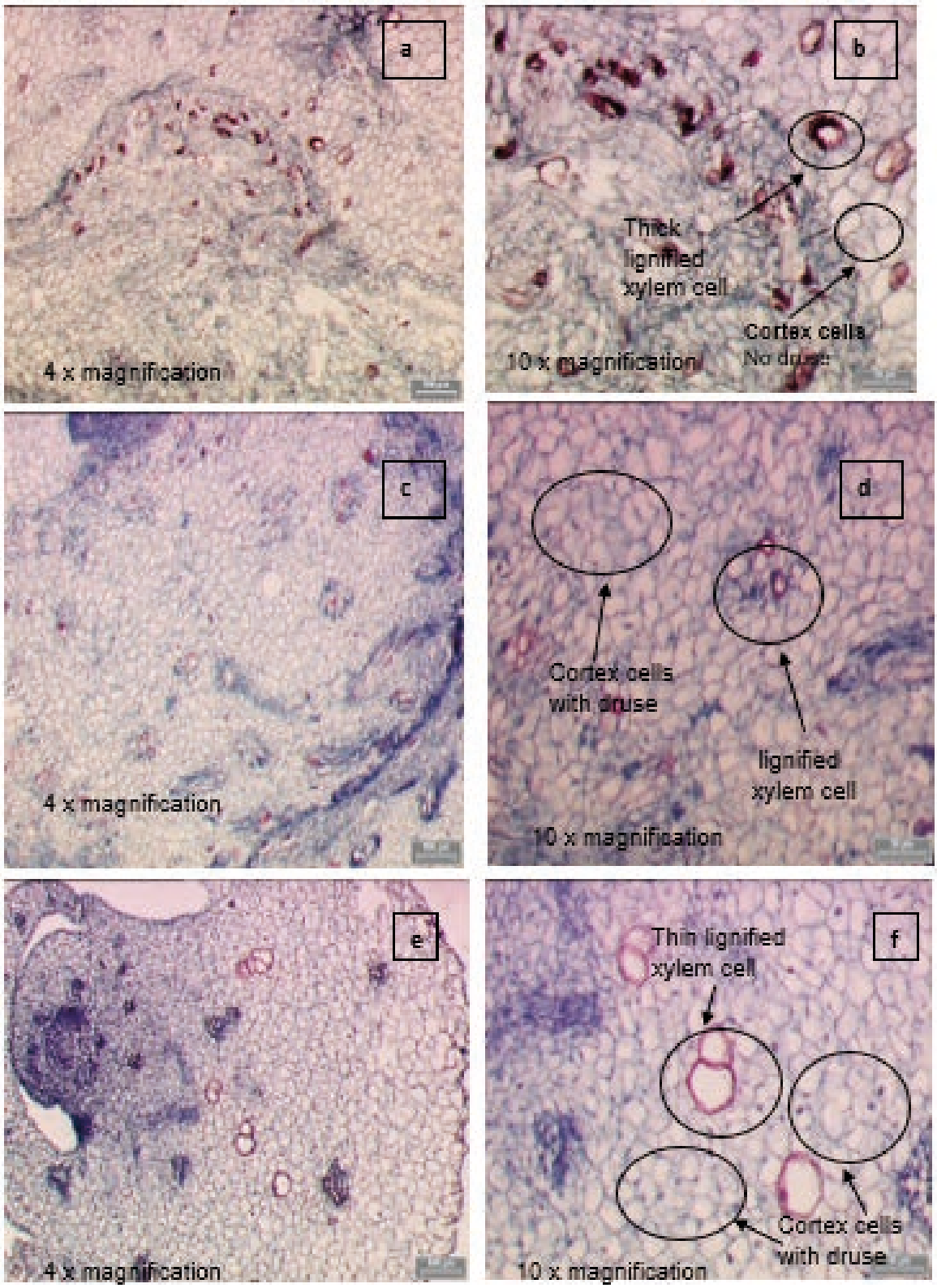

Figure 2: Histology images of basal pseudostem of *C. mangga* plantlets cultured in proliferation medium (Control) at 4 x optical magnification (a) and 10 x optical magnification (b); Proliferation medium supplemented with 3.5 mgL^{-1} YE at 4 x optical magnification (c) and 10x optical magnification (d); Proliferation medium supplemented with 5.0 $mg\ l^{-1}$ YE at 4 x optical magnification (e) and 10x optical magnification (f)

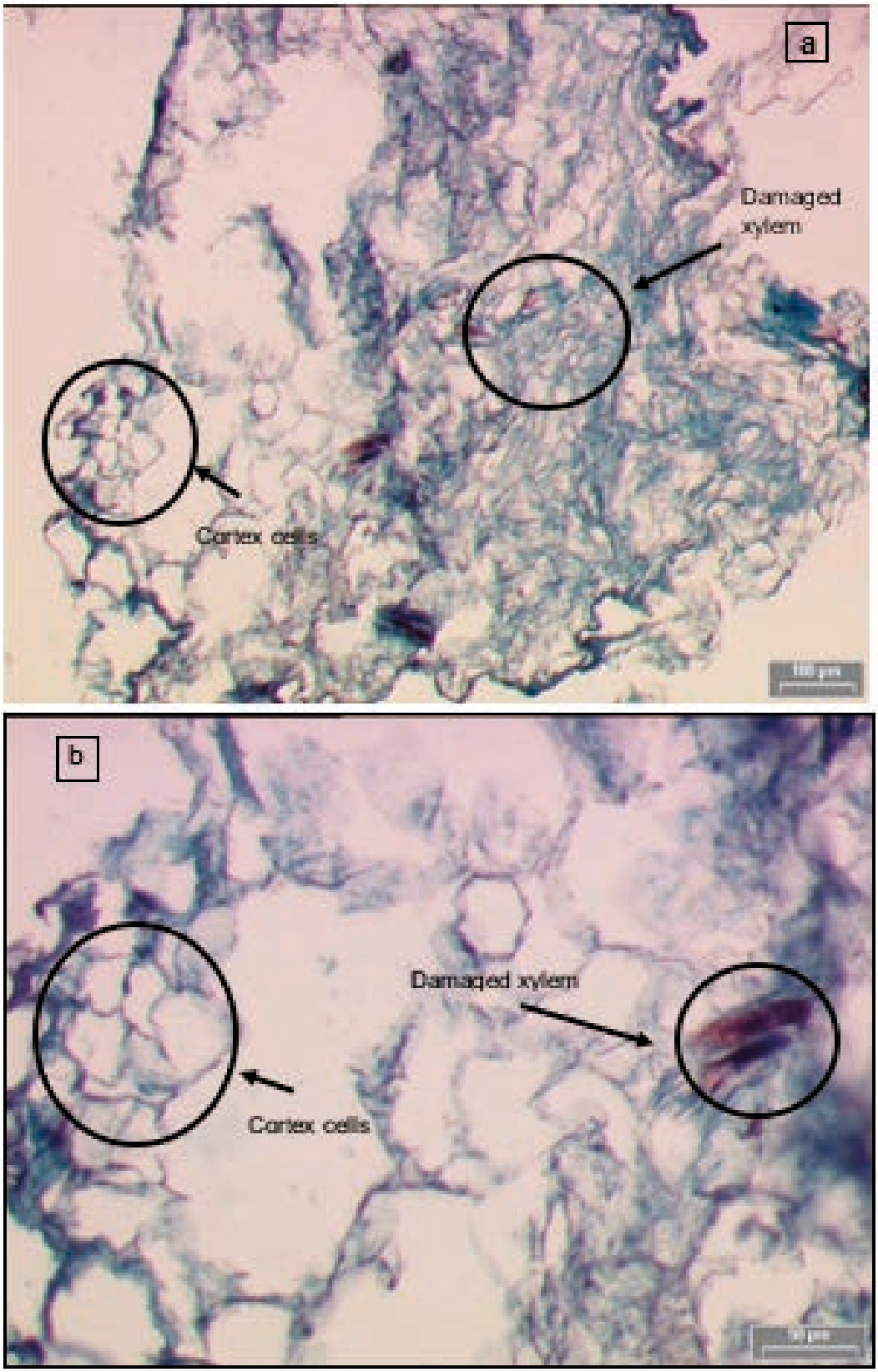

Figure 3: Histology images of basal pseudostem of *C. mangga* plantlets cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract at 4x optical magnification (a) and 10x optical magnification (b)

1980; Webb, 1999; Nakata, 2012). Even though oxalate crystals were often found in higher plant, the formation and function of oxalate crystals in plants were still unclear (Webb, 1999). It was proposed that oxalate crystal might be involved in ion balance, plant defence, tissue rigidity and support, detoxification, light accumulator, and reflector (Franceschi and Homer, 1980; Doege, 2003). The possible function of oxalate crystal as part of plant defence might be relevant in *C. mangga* study. The oxalate crystal could not be detected in plantlets cultured in medium without elicitor (control) (Figure 2 a & b). Most part of the tissue of *C. mangga* plantlets cultured in medium supplemented with 150 mg l^{-1} chitosan and 3.5 mg l^{-1} yeast extract were damaged during dehydration process of histology preparation. The damaged tissue indirectly indicate that the cell walls of those plantlets were more delicate than the cell walls of plantlets cultured in medium supplemented with yeast extract or the control. This finding was also supported the observation on the visual morphological characteristic of those plantlets which exhibited brittleness on the abnormal formed shoots cultured in proliferation medium supplemented with 150 mg l^{-1} chitosan plus 3.5 mg l^{-1} yeast extract.

Chitosan has been used to study the defence mechanism of plants towards their fungal pathogens. Unlike yeast extract, chitosan is a compound with defined molecular structure (polycationic b-1,4-linked-d-glucosamine polymers) which resemble to cell wall component of fungi (Walker–Simmons et al., 1983). The interaction between oligosaccharins and receptors located in the plant membranes results in the production of many plants defence secondary metabolites and many phenolic and terpenoid compounds (Kim and Lee 2011; Pang et al 2021). It was found to induce the synthesis of pathogenesis-related (PR) proteins and several defence enzymes (such as phenylalanine ammonia lyase and peroxidase) (Riaz et al., 2014). The plant defence compounds, synthesized after chitosan elicitation, have relevant physiological activity, mainly as antioxidants (the polyphenols), might have resulted in cell damage as well.

4 CONCLUSIONS

Longitudinally half incised shoot explants and cultured vertically on shoot proliferation medium, MS supplemented with 2.0 mg l^{-1} benzyl adenine (BA) and 0.5 mg l^{-1} NAA, promote shoot proliferation by 100 %. This method can be used as an alternative technique for multiplication of the *in vitro* plantlets of *C. mangga* which normally propagate at a slow rate. Supplementation of 3.5 or 5.0 mg l^{-1} yeast extract, or combination of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan, into the shoot proliferation medium could be used as an alternative mode for enhancing anti-lipid peroxidation activity. However, biotic elicitation with 3.5 or 5.0 mg l^{-1} yeast extract, or combination of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan did not affect the cortex cell size but a reduction in xylem cell numbers of *C. mangga* pseudostem. Supplementation of 5.0 mg l^{-1} yeast extract reduced the size of the xylem cell by more than half as compared to the xylem cell of those cultured in proliferation supplemented with 3.5 mg l^{-1} yeast extract and the control. The addition of 3.5 mg l^{-1} yeast extract and 150 mg l^{-1} chitosan into the proliferation medium resulted in abnormal anatomy of their pseudostems with damage of the xylem cells.

5 REFERENCES

Abbas, M. S., Taha, H. S., Aly, U. I., El-Shabrawi, H. M., Gaber, E. S. I. (2011). In vitro propagation of ginger (*Zingiber officinale* Rosco). *Journal of Genetic Engineering and Biotechnology*, *9*(2), 165–172. https://doi.org/10.1016/j.jgeb.2011.11.002

Abraham, F. (2010). *Elicitation effect on the growth and biochemical activities of Curcuma mangga* Val. *(*Zingiberaceae*) in vitro plantlets*. Master of Science Thesis, Universiti Sains Malaysia, Penang, Malaysia.

Abraham, F., Bhatt, A., Chan, L.K., Indrayanto, G., Sulaiman, S. F. (2011). Effect of yeast extract and chitosan on shoot proliferation, morphology and antioxidant activity of *Curcuma mangga* in vitro plantlets. *African Journal of Biotechnology*, *10*(40), 7787–7795. https://doi.org/10.5897/ajb10.1261

Ahmed Hasan, S., Khasim, S.M., Ramudu, J. (2020). Development of Standard Protocols for In Vitro Regeneration of Some Selected Banana Cultivars (Musa spp.) from India. In: Khasim, S.M., Long, C., Thammasiri, K., Lutken, H. (eds) *Medicinal Plants: Biodiversity, Sustainable Utilization and Conservation*. Springer, Singapore. https://doi.org/10.1007/978-981-15-1636-8_45

Awin, T., Mediani, A., Mohd Faudzi, S. M., Maulidiani, Leong, S. W., Shaari, K., Abas, F. (2020). Identification of α-glucosidase inhibitory compounds from *Curcuma mangga* fractions. *International Journal of Food Properties*, *23*(1), 154–166. https://doi.org/10.1080/10942912.2020.1716792

Bejoy, M., Dan, M., Anish, N. P., Anjana, R. G., Radhika, B.J., Manesh, K. (2012). Micropropagation of Indian ginger (*Curcuma vamana* Sabu & Mangaly): A wild relative of tumeric. *Biotechnology*, *11*(6), 333–338. https://doi.org/10.3923/biotech.2012.333.338

David, A.V.A., Arulmoli, R., Parasuraman, S. (2016). Overviews of biological importance of quercetin: a bioactive flavonoid. *Pharmacognosy Reviews*, *10*, 84-89. https://doi.org/10.4103/0973-7847.194044

Doege, S. J. (2003). The role of natural calcium oxalate crystals in plant defense against chewing insects. Inquiry: *The University of Arkansas Undergraduate Research Journal*, *4*, 88–94.

El-Hawaz, R.F., Bridges, W.C., Adelberg, J.W. (2015). In vitro growth of *Curcuma longa* L. in response to five mineral elements and plant density in fed-batch culture systems. *PLOS One*, *10*(4), 1-13. http://doi10.1371/journal-pone.0118912

Espinosa-Leal, C.A., Puente-Garza, C.A., Garcia-Lara, S. (2018). *In vitro* plant tissue culture: means for production of biological active compounds. *Planta*, *248*, 1-18. https://doi.org/10.1007/s00425-018-2910-1

Farjaminezhad, R. and Garoosi, G. (2021). Improvement and prediction of secondary metabolites production under yeast extract elicitation of *Azadirachta indica* cell suspension culture using response surface methodology. *AMB Express, 11*, 1-16. https://doi.org/10.1186/s13568-021-01203-x

Ferrari, M. P. de S., Antoniazzi, D., Nascimento, A. B., Franz, L. F., Bezerra, C. S., Magalhães, H. M. (2016). Evaluation of new protocols to *Curcuma longa* micropropagation: A medicinal and ornamental specie. *Journal of Medicinal Plants Research*, *10*(25), 367–376. https://doi.org/10.5897/jmpr2016.6109

Fitriastuti, D., Julianto, T.S., Iman, A. W. N. (2020). Identification and heme polymerization inhibition activity (HPIA) assay of ethanolic extract and fraction of Temu Mangga (*Curcuma mangga* Val.) rhizome. *EKSAKTA: Journal of Sciences and Data Analysis*, *1*(1), 64–72. https://doi.org/10.20885/eksakta.vol1.iss1.art10

Franceschi, V.R. and Horner, H.T. (1980). Calcium oxalate crystals in plants. *The Botanical Review*, *46*, 361–427. https://doi.org/10.1007/BF02860532

Freeman DJ, Wenerstrom G, Spruance SL. (1985).Treatment of recurrent herpes simplex labialis with topical butylated hydroxytoluene. *Clinical Pharmacology & Therapeutics*, *38*(1), 56-9. https://doi.org/10.1038/clpt.1985.134

Furmuly, A. M., Azemi, N. (2020). A review on golden species of Zingiberaceae family: Genus *Curcuma*. *Journal of Critical Reviews*, *7*(16), 882–895. https://doi.org/10.31838/jcr.07.16.114

Hedayati, A., Hemmaty, S., Nourozi, E. , Amirsadeghi, A. (2021) Effect of yeast extract on the gene expression and tropane alkaloids production in *Atropa belladonna* L. hairy roots. *Russian Journal of Plant Physiology*, *68*, 102–109 (2021). https://doi.org/10.1134/S1021443721010088

Jala, A. (2012). Effects of NAA BA and sucrose on shoot induction and rapid micropropagation by trimming shoot of *Curcuma longa* L. *International Transaction Journal of Engineering, Management, & Applied Sciences & Technologies*, *3*(2), 101–109. http://TuEngr.com/V03/101-109.pdf.

Leong-Škorničková, J., Soonthornkalump, S., Thongbai, W. (2021). Four new *Curcuma* species (Zingiberaceae) from Thailand. *Blumea*, *65*, 244-253. https://doi.org/10.3767/blumea.2021.65.03.09

Kim, Y.O. and Lee, E.J. (2011). Comparison of phenolic compounds and the effects of invasive and native species in East Asia: support for the novel weapons hypothesis. *Ecological Research*, *26*, 87–94. https://doi.org/10.1007/s11284-010-0762-7

Kochan, E., Szymczyk, P., Kuzma, L., Lipert, A., Szymanska, G. (2017). Yeast extract stimulates ginsenoside production in hairy root cultures of American ginseng cultivated in shake flasks and nutrient sprinkle bioreactors. *Molecules*, *22*, 1-15. https://doi.org/10.3390/molecules22060880

Li, Y.Y., Tian, Q.L., Yu, H.W., Lu, L.M. (2021). Progress towards a molecular-level understanding of *Curcuma alismatifolia*. *European Journal of Horticultural Science*, *86*(3), 328-334. https://doi.org/10.17660/eJHS.2021/86.3.12

Marchant, M.J., Molina, P., Montecinos, M., Guzman, L., Balada, C., Fassio., C., Castro, M. (2021). *In vitro* propagation of Easter Islaand *Curcuma longa* from rhizome explants using temporary immersion system. *Agronomy*, *2121*, 1-11. http://doi.org/10.3390/agronomy11112121

Maryam, S. and Martiningsih (2021). Antioxidant activity and total fenol content white saffron (*Curcuma mangga* Val). *IOP Conference Series: Materials Science and Engineering*, *1115*(1), 012081. https://doi.org/10.1088/1757-899x/1115/1/012081

Mohanty, S., Parida, R., Sandeep,I., Sahoo, S., Nayak, S. (2015). Evaluation of drug yielding potential of micropropagated *Curcuma aromatica*. *International Journal of Pharmacy and Pharmaceutical Sciences*, *7*(4), 71-76.

Mok., P.K. and Ho., W.S. (2019). Rapid in vitro propagation and efficient acclimatization of *Neolamarckia cadamba*. *Asian Journal of Plant Science*, *18*, 153-163. https://doi.org/10.3923/ajps.2019.153.163

Muchtaromah, B., Ahmad, M., Romaidi, Nazilah, L. A., Naja, N. A. (2018). Antibacterial activity of water and ethanol extract of *Allium sativum*, *Curcuma mangga*, and *Acorus calamus* combination. *Journal of Biological Researches*, *24*(1), 8–15. https://doi.org/10.23869/bphjbr.24.1.20182

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nakata, P.A. (2012). Plant calcium oxalate crystal formation, function, and its impact on human health. Frontiers of Biology, *7*, 254–266. https://doi.org/10.1007/s11515-012-1224-0

Osawa, T. and Namiki, M. (1981). A novel type of antioxidant isolated from leaf wax of Eucalyptus leaves. *Agricultural and Biological Chemistry*, *45*(3), 735–739. https://doi.org/10.1080/00021369.1981.10864583

Pang, Z., Chen, J., Wang, T., Gao, C., Li, Z. Guo, L., Xu, J., Cheng, Yi. (2021). Linking plant secondary metabolites and plant microbiomes: a review. *Frontiers in Plant Science*, *22*, 1-22. https://doi.org/10.3389/fpls.2021.621276

Pikulthong, V., Teerakathiti, T., Thamchaipenet, A., Peyachoknagul, S. (2016). Development of somatic embryos for genetic transformation in *Curcuma longa* L. and *Curcuma mangga* Valeton & Zijp. *Agriculture and Natural Resources*, *50*(4), 276-285. https://doi.org/10.1016/j.anres.2015.08.004

Ramadanil, Damry, Rusdi, Hamzah, B., Zubair, M. S. (2019). Traditional usages and phytochemical screenings of selected Zingiberaceae from central Sulawesi, Indonesia.

Pharmacognosy Journal, *11*(3), 505–510. https://doi.org/10.5530/pj.2019.11.80

Riaz, A., Riaz, A., Rattu, A.U.R., Tahir, M.I., Azeem, M. (2014). Phenylalanine ammonia-lyse (PAL) and peroxidase activity in brown rust infected tissues of Pakistani wheat cultivars. *Pakistan Journal of Botany*, *46*(3), 1101-1107. https://www.researchgate.net/publication/262896801

Sánchez-Sampedro, M. A., Fernández-Tárrago, J., Corchete, P. (2005). Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. *Journal of Biotechnology*, *119*(1), 60–69. https://doi.org/10.1016/j.jbiotec.2005.06.012

Sharmin, S.A., Alam, M.J., Sheikh, M.M.I., Zaman, R., Khalekuzzaman, M., Mondal, S.C., Haque, M.A., Alam, M.F., Alam, I. (2013). Micropropagation and antimicrobial activity of *Curcuma aromatica* Salisb., a threatened aromatic medicinal plant. *Turkish Journal of Biology*, *37*, 698-708. http://doi:10.3906/biy-1212-11

Škorničková, J. (2007). *Taxonomic Studies in Indian Curcuma* L. Ph.D Thesis, Charles University, Prague, Czech Republic.

Soonthornkalump, S., Ongsakul, A., Dolaji, A., Leong-Škorničková, J. (2020). *Curcuma papilionacea* (Zingiberaceae), an unusual new species from southern Thailand. *Phytotaxa*, *432*(1), 011-016. https://doi.org/10.11646/phytotaxa.432.1.2

Subositi, D. and Wahyono, S. (2019). Study of the genus curcuma in Indonesia used as traditional herbal medicines. *Biodiversitas*, *20*(5), 1356–1361. https://doi.org/10.13057/biodiv/d200527

Sudipta, J., Asit, R., Ambika, S., Suprava, S., Biswabhusan, D., Basudeba, K., Sanghamitra, N. (2020). Rapid plant regeneration in industrially important *Curcuma zedoaria* revealing genetic and biochemical fidelity of the regenerants. *3 Biotech, 10*, 17. https://doi.org/10.1007/s13205-019-2009-9

Walker-Simmons, M., Hadwiger, L., Ryan, C.A. (1983). Chitosans and pectic polysaccharides both induce the accumulation of the antifungal phytoalexin pisatin in pea pods and antinutrient proteinase inhibitors in tomato leaves. *Biochemical and Biophysical Research Communications*, *110*(1), 194-199. http://10.1016/0006-291x(83)91279-2

Webb, M. A. (1999). Cell-mediated crystallization of calcium oxalate in plants. *Plant Cell*, *11*(4), 751–761. https://doi.org/10.1105/tpc.11.4.751

Yuandani, Yuliasmi, S., Satria, D., Dongoran, R. F., Sinaga, M. S., Marpaung, N. H. A. (2019). Correlation between the phytochemical constituents of *Curcuma mangga* and its immunomodulatory effect. *Rasayan Journal of Chemistry*, *12*(1), 1–6. https://doi.org/10.31788/RJC.2019.1215050

Zahid, N. A., Jaafar, H. Z., Hakiman, M. (2021). Micropropagation of ginger (Zingiber officinale 'Bentong') and evaluation of its secondary metabolites and antioxidant activities compared with the conventionally propagated plant. *Plants*, *10*, 1–17. https://doi.org/10.3390/plants10040630