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Optimizing of root induction in oil palm plantlets for acclimatization by some potent plant growth regulators (PGRs)

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Embryo derived shoots (EDS) of oil palm from culturing young leaves of mature trees were rooted in various culture media which were supplemented with different concentrations of ∝-naphthalene acetic acid (NAA) (0, 2, 4, 6, 8 mg/l) and paclobutrazol (PBZ) (0, 3, 6, 9, 12 mg/l). After culture for 8-10 weeks morphological, physiological and anatomical of shoots, leaves, and roots were examined. The results revealed that Woody Plant medium (WPM) supplemented with 7.2% sucrose, 6 mg/l NAA and 9 mg/l PBZ gave the best results for all parameters. A maximum number of shoot at 2 shoots/cultured shoot was obtained after 6 weeks of culture. Morphological characters; shoot length (cm) and diameter (cm), leaf number (5.7 leaves/shoot) and leaf width (1.2 cm) were maximum. The greatest fibrous thickening root induction frequency was obtained at 88%. Anatomical study of leaf revealed that PBZ-treated shoot provided an increment in epicuticular layer. PBZ-treated plantlets were healthy with dark green leaves which showed the highest total chlorophyll content at 3.54 mg/ g fresh weight (FW).

Key words: embryo derived shoots, oil palm (*Elaeis quineensis* Jacq.), paclobutrazol, root induction

Introduction

Oil palm is an arborescent monocotyledon and cannot be multiplied by conventional means of vegetative propagation. Success in plant regeneration by means of somatic embryogenesis has already been reported (Rabechault *et al.*, 1974). Much research has been carried out from somatic embryo cultures in oil palm. The establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory and micropropagation has also been identified as suitable alternative for rapid and large-scale plant production (Khaw and Ng, 1998). However, several reports *in vitro* multiplication of oil palm is associated with difficulties in different stages of micropropagation particularly high-frequency rooting of micro-shoots leading to failness of roots formation (Kanchanapoom and

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Domyoas, 1999). Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of oil palm, including many other plant species. Plantlets that have grown *in vitro* have been continuously exposed to a unique microenvironment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing simple sugar and nutrients to allow for heterotrophic growth and in atmosphere with high level of humidity make them survive at low frequency of survival just after transfer to soil (Kadlecek *et al*., 2001). To improve survival rate of the vitro plant to soil or field conditions the use of chemical such as plant growth regulators are of great interested. Moreover, the recent development of highly active growth retardants has further enhanced the potential uses of chemical regulators. Among them, paclobutrazol (PBZ) is widely used (Fernandes *et al*., 2004). However, so far, substantial information on the *in vitro* rooting and acclimatization of oil palm shoot has been shown very rare.

Growth regulators have been reported to improve the quality of vitro-plant. Optimization of growth regulator related to enhance chlorophyll and carotenoid content of leaf and could be effected to stimulatory or regulatory action on biosynthesis of chloroplast pigments (Iqtidar *et al.*, 1994). The ability of PBZ to induce root from vitro-plant has not yet been reported in oil palm or other palm species. Thus, it's the first report to describe the effect of PBZ on root induction of vitro-shoots of oil palm. The advantages would great important in commercial propagation of oil palm through tissue culture technique. Therefore, the objective of this study was to develop a protocol for high frequency of root induction from vitro-shoots and acclimatization plantlets to soil conditions. The main activities were focus on the investigation of PBZ with NAA on the production of micropropagated oil palm during acclimatization stage. Moreover, the effect of PBZ onto enhance transplanting success to soil and particularly high frequency rooting of microshoots of oil palm.

Materials and methods

Plant material

Plant material used in this research study was embryo derived shoots (EDS) of oil palm. Those shoots were derived from culturing young leaves of mature trees taken from Thepa Research Station, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. Primary callus and embryogenic callus were induced by the protocol of Te-chato *et al.* (2004) and the callus was maintained on embryogenic callus proliferation medium for at

least two years (subculture monthly intervals). Vitro-shoots developed on regeneration medium were used for root induction both *in vitro* and *ex vitro*.

Explants preparation and treatments

Vitro-shoots were removed from culture vessel and transferred to three different culture media namely, ½MS, MS and WPM. All the culture media were supplemented with different concentrations of NAA (0, 2, 4, 6 and 8 mg/l) and PBZ (0, 3, 6, 9 and 12 mg/l). All above treatments consisted of three replications and two shoots per replication. The observed data were recorded by SAS 6.0 (Statistically Analysis System).

Effect of culture media, NAA and PBZ on morphology and root induction

Two factors; culture media and PGRs were investigated for root induction. Three kinds of culture media; ½MS, MS and WPM were used and all culture media were supplemented with 7.2% sucrose. Each culture medium was supplemented with NAA (0, 2, 4, 6 and 8 mg/l) and PBZ (0, 3, 6, 9 and 12 mg/l). All media were adjusted pH to 5.7 before autoclaving and solidified with 0.6% agar. The cultures were maintained under 14 h photoperiod of 25 µmol/m^2/s at 28±1^{o}C. After culture for 4-6 weeks percentage of shoot produced root, number of roots and root length were recorded and statistically compared in each factor separately using completely randomized design (CRD). Twenty shoots were used for each treatment. Complete plantlets (shoots with roots) obtained from the above procedures were carefully pulled out from test tube and agar-agar was absolutely removed. The plantlets were placed in 4 inch black polybag containing soil mixture and kept under high humidity and low light intensity. During 4 weeks of acclimatization under gradually decrease in humidity and gradually increase light intensity, survival percentage of plantlets obtained from each culture media and PGRs were recorded and statistically compared using CRD.

Anatomical study of leaf and root

After three months of growth leaves and roots from complete plantlet *in vitro* were collected. At least three representative plants per treatment were collected. Sampling was carried out according to the following procedure; leaf and root sample were sliced in thin section using sharp lazor blade. Those segments were placed on slide glass with a drop of distilled water. The slides were then covered with cover slip and observed under compound microscopy. Anatomical characteristics of cells in each layer were recorded and compared among the plants or samples in different culture media and PGRs.

Chlorophyll content analysis

Leaf sample of vitro-shoot in different concentration of NAA and PBZ containing MS medium from each culture medium supplemented with different kinds and concentrations of plant growth regulators at different culture period were collected. Leaf samples were ground in a mixture solution of 1% acetone and 5 ml methanol. The solution was leaved overnight at 4^{o}C then centrifuged at 1000 rpm for 10 min. The supernatant was brought to measure OD_{650} and OD_{665} by spectrophotometer and quantity of chlorophyll a, b and total chlorophyll (a+b) per unit leaf area were estimated (Zhang *et al.,* 2002). Each three consecutive leaves were taken in basipetal sequence from the youngest expanded leaves and a mean value was obtained for each plantlet.

Results and discussion

Effect of culture media, NAA and PBZ on morphology and root induction

Multiple shoot formation and morphology of shoot

Combination of NAA and PBZ could induce multiple shoot formation. These hormonal combinations showed effective on induction of direct multiple shoot formation. The current study revealed that the two hormones promoted *in vitro* shoot multiplication from shoot vitro of oil palm (shoot without root) (Fig. 1A). Shoot proliferation was observed after 6 weeks of culture. According to ANOVA, maximum number of shoot at 2 shoots/cultured shoot was obtained from 6 mg/l NAA and 9 mg/l PBZ containing WPM medium, significant difference to other concentrations. However, multiple shoot formation was obtained from all treatments, indicating that the combination of NAA and PBZ containing WPM were the most suitable among half strength and full strength MS.

PBZ is azole derivative which had been found to promote the shoot inducing capability. PBZ at low concentration of 3 mg/l gave the highest shoot number of 3.5 in korarima (*Aframomum corrorima* (Braun) Jansen) (Tefera and Wannakrairoj, 2006) while a very low concentration at 0.05-0.075 mg/l gave the best result in Friederick's Dendrobium (Te-chato *et al.*, unpublished data). In this present study, it was found that slightly high concentration of PBZ (9 mg/l) was effective on shoot induction. In case of herbaceous gloxinia, it could tolerate to very high concentration of PBZ at 500 mg/l (Te-cahto and Chudecha, 2006). The different response to concentration of PBZ might be expressed species specific. In addition, physiological status of sources of explants (excised single shoot or shoot with few nodes) used for culture was different lead to the difference in balancing of indigenous phytohormones. Apical buds or shoot apex of cultured shoot were promoted to develop a new emerging shoot at an

average of 2.2 shoots/cultured shoot (Fig. 1A). Some authors reported that PBZ enhanced cytokinin activities and induced adventitious shoot proliferation in Araceae (Werbrouck and Debergh, 1996). In addition, synergistic effects of PBZ and cytokinins, especially BA or TDZ were enhanced on shoot proliferation (Werbrouck and Debergh, 1996; Tefera and Wannakrairoj, 2006). Unfortunately, cytokinins were not systematically used in combination with PBZ in this experiment. So, their synergistic effect on proliferation of oil palm in this study can not be postulated.

Plant growth retardants generally reduce elongation of the internodes of higher plants both *in vitro* and *ex vitro*. In case of stem length all PBZ-treated plants gave shorter internode due to the inhibitory effect on gibberellic acid production. It is active as a growth retardant in broad spectrum of species (Dalziel and Lawence, 1984; Lever *et al.*, 1982) especially in *Chrysanthemum x morifolium* (Barrett, 1982; McDaniel, 1983; Menhenett, 1984). Contrary result was obtained in this present study. Oil palm shoot cultured in WPM in the presence of PBZ showed higher length of shoot. PBZ at concentration of 9 mg/l in combination with 6 mg/l NAA promoted the highest shoot length at 11.4 cm, significant difference to other concentrations (Fig. 1B). For diameter of stem or stem width all shoots treat with PBZ gave higher diameter of stem. Among concentration tested 3-9 mg/l PBZ together with 2-8 mg/l NAA gave significant diameter of stem higher than other concentrations (Fig. 1C). The changes that PBZ application induced in various plant parts, especially larger diameter of stem might be due to the increment in width of bundle sheath or storage of starch in parenchymatous tissue. Similar result was also described by Aguirre and Blanco (1990). However, McDaniel *et al.* (1990) found that PBZ caused a weak stem.

The development of leaves obtained from various treatments of PBZ and NAA was quite different. This positive effect on leaf number became significant difference from the fourth week after culture. At the end of the experimental period, calculation of the total number of leaves which was developed during 6 weeks confirmed the significant effect of various concentrations NAA and PBZ on plant growth during this stage. Mostly, the interaction of two main factors showed no significant difference at early time of *in vitro* cultured. All plantlets gave nearly the same number of the leaves. Emergence of the new forming leaves developed after 2 weeks of culture, but significant differences was not observed. Most of shoots developed new forming leaf after 6 weeks of culture. The highest number of leaves was obtained from WPM supplemented with 6 mg/l NAA and 9 mg/l PBZ at 5.7 leaves/shoot, significant difference ($p<0.05$) from the other treatments (Fig. 2 A). Only few authors reported the effect of PBZ on leaf number per plant. In

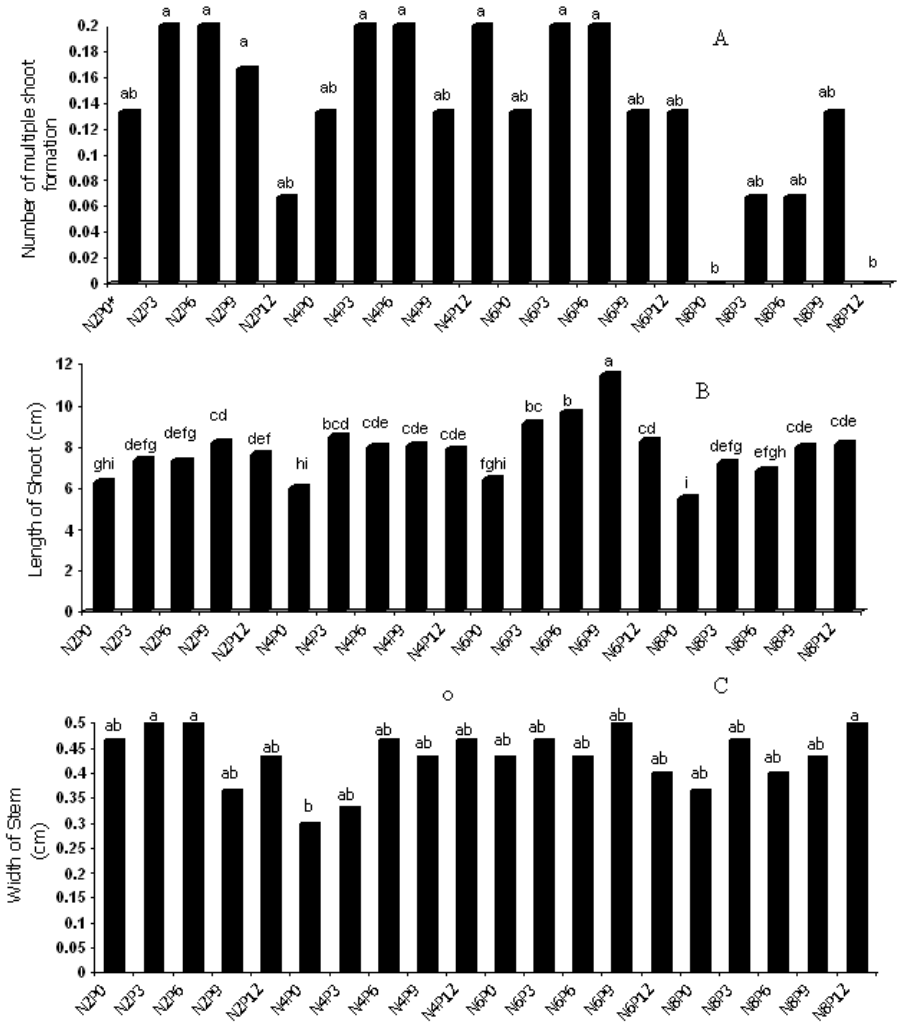
case of chrysanthemum, a low concentration at 0.5 mg/l promoted leaf number whereas higher concentration decreased those number (Smith *et al.*, 1990).

The maximum mean width of leaves at 1.20 cm was obtained on WPM supplemented with 6mg/l NAA and 9 mg/l PBZ, significant difference ($p<0.05$) to other combinations of NAA and PBZ (Fig. 2B). The leaves of treated shoot were thicker than those of control. The increased thickness might be due to increase in palisade and spongy mesophyll thickness like the report of Burrow *et al.* (1992). On the other hand, PBZ increased epicuticular wax which closely related to leaf thickness.

Root induction

In vitro shoot of oil palm were able to form root when cultured on WPM supplemented with various concentrations of NAA and PBZ. The greatest rooting percentage was obtained using WPM supplemented with 6 mg/l NAA and 9 mg/l PBZ at 88% (Figs. 3, 4). Without NAA and PBZ only a short single root developed (Fig. 5A). The average number of root formed from each shoot and their length increased as the concentration NAA and PBZ increased. The most effective concentration of NAA and PBZ gave vigorous and healthy with extensive growth of roots was 6 mg/l NAA and 9 mg/l PBZ (Fig. 5B). In this hormonal combination fibrous root system was obtained. PBZ and NAA at concentrations 8 mg/l and 12 mg/l gave a thick and stumpy of root and caused a severe stunt of shoot growth (Fig. 5C). So, it is suggest that high concentration of NAA and PBZ were not suitable for root induction. From our previous studies root induction form excised single shoot in MS medium with 3 mg/l NAA and 1 mg/l 2i-P that was never exceed 40%. By modification culture medium with low concentration of NAA and BA, root induction percentage increased to nearly 75 (Te-chato and Muangkaewngam, 1992; Te-chato, 1998). Thus, the present study indicated that NAA and PBZ at optimum concentration in WPM medium with high concentration of sucrose was able to induce root better than MS medium and low concentration of sucrose.

The ability to produce roots is important for successful micropropagation. A positive result in healthy growth of root formation gave the high rate of survival after transferring to soil or field condition. Besides of plant regulators treatment, sucrose level in medium was played an important role in growth and development of root formation (Fuentes *et al.*, 2005).



* 2 mg/l NAA and 0 mg/l PBZ

Fig. 1. Effect of NAA and PBZ containing MS medium on formation and morphological characters of *in vitro* shoot.

Anatomical study of leaf and root

The first indication of vigor vitro-plant affected by PBZ is the presence of cell changing in anatomy of leaves. New forming leaves developed from vitro-shoots were thick and wide, so called juvenile leaves, whereas older leaves were referred to as adult leaves. This difference related not only leaf size but also the internal structure. In fact, leaves from untreated plants showed a thin-wall upper epidermis with a very thin cuticle the leaves of treated plants which

were a darker green and more thick than those of control (Fig. 6). Similar result was also reported in treated with PBZ (Burrow *et al.*,1992). However, increased thickness in *Chrysanthemum* leaves was due to increases in palisade (64%) and spongy mesophyll (72%) whereas increased thickness in oil palm leaves in this present study was due to increase in cuticle layer and bundle sheath. Individual bundle sheath cells were larger or bigger in the PBZ-treated shoots (Fig. 6) together with two to three layers of cuticle resulted in the thicker leaves.

It was clearly shown that leaf treated by PGRs were affected for mitochondria of the bundle sheath cells, which are much larger than those of adjacent mesophyll cells, may provide some of the energy for this transport system of photosynthetic product. Metabolically, which large size of bundle sheath cells much more active than those of surrounding of mesophyll.

Control plants possessed tap root system, while PBZ-treated plants showed fibrous root system. Low concentration of PBZ (3-6 mg/l) gave a medium size of fibrous roots whereas high concentration (12 mg/l) expressed many large diameter roots (Fig. 7). The medium fibrous thickening of the roots of PBZ-treated plants is common phenomena (Barnes *et al.,* 1989; Bausher and Yolenosky, 1987; Te-chato and Nujeen, 2007) and inhibition of lateral roots has also been reported (Bausher and Yolenosky, 1987). The result supports to these observation and suggests that whether or not the metabolism of endogenous cytokinins is influenced, especially cell division and enlargement. The number of roots and their thickness were greatly increased at particular high concentration of PBZ.

Chlorophyll content analysis

The chlorophyll content of the leaves increased with the age of the plant. The increased concentration of either NAA or PBZ promoted significant increasing in chlorophyll content. The highest total chlorophyll content was obtained from 6 mg/l NAA and 9 mg/l PBZ at 3.54 mg/g FW. On the other hand, low total chorophyll contents were found on control plant at 1.94 mg/g FW. Combination NAA and PBZ treatments increased the chlorophyll content when compared to control (Fig. 4). PBZ treated shoots gave nearly two times more chlorophyll than control. Similar results were also reported in barley seedling (Sakar *et al.*, 2004) and tomato (Still and Pill, 2004). New forming leaves from PBZ treated shoot were dark green due to high chlorophyll a, b and total chlorophyll. Fletcher *et al.* (2000) proposed that PBZ as one in triazol group stimulate cytokinin synthesis that enhances chloroplast differentiation, chlorophyll biosynthesis and prevents chlorophyll degradation. Moreover, Te-chato *et al.* (2008) proved that KN was necessary for generation of chlorophyll in oil palm cell suspension culture. The mechanism of KN or cytokinin on

chloroplast formation was not clearly understood. It might involve in cell division and some protein synthesis in relation with chloroplast development. It is suggested that PBZ may involve in the formation of cytokinin, especially kinetin

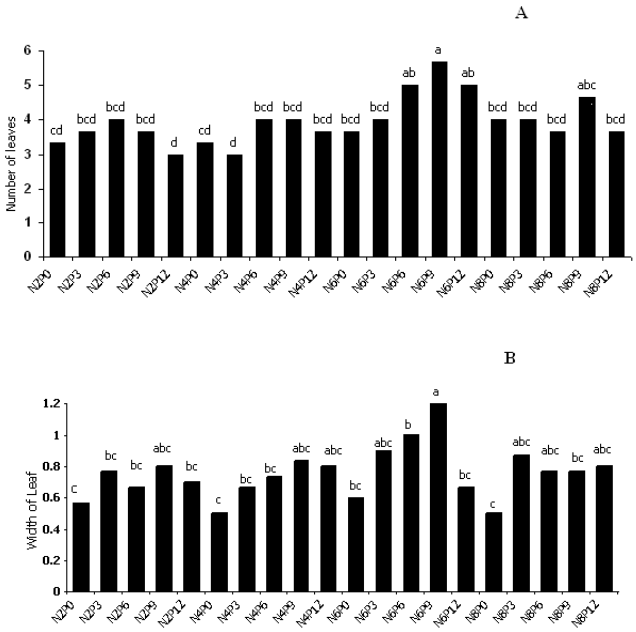


Fig. 2. Effect of NAA and PBZ containing MS medium on a number and width of leaves of *in vitro* shoot.

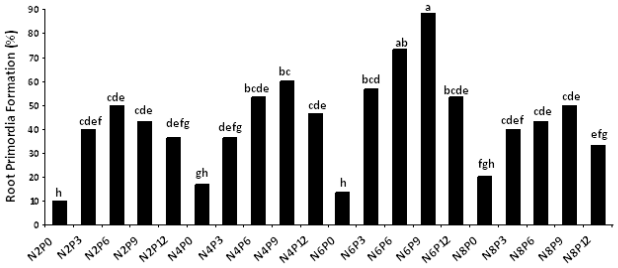


Fig. 3. Effect of NAA and PBZ containing MS medium on root formation percentage.

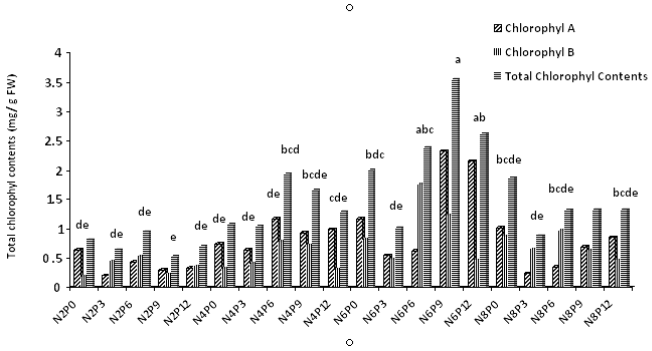


Fig. 4. Effect of NAA and PBZ containing MS medium on accumulation of chlorophyll in leaves.

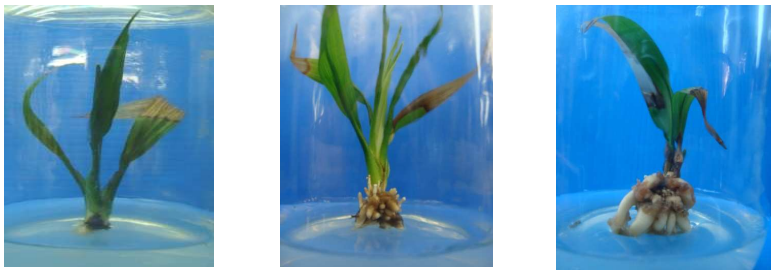


Fig. 5. Root formation from excised single shoot in WPM without PGR (A), 6 mg/l NAA and 9 mg/l PBZ (B) and 8 mg/l NAA and 12 mg/l PBZ (C) after 8 weeks of culture.

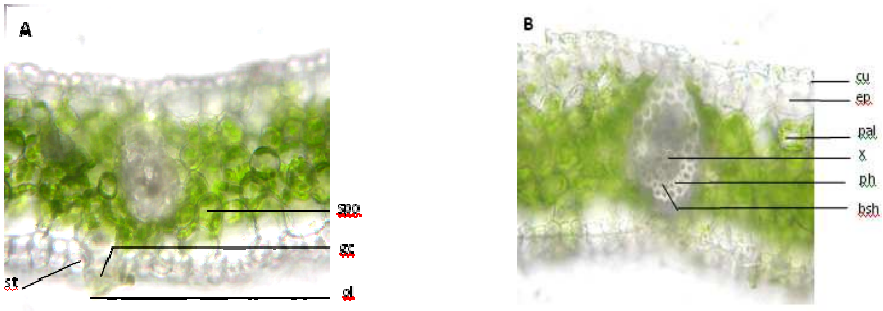


Fig. 6. Transverse section of vitro-leaf plant oil palm. A: control. B: leaf treated with 6 mg/l NAA and 9 mg/l PBZ. Both sources of leaves obtained from shoots in WPM for 8 weeks. Abbreviation : bsh, vascular bundle sheath; cu, cuticle; epi, epidermis; gc, guard cell; ol, outer ledge; pal, palisade cell; ph, phloem; spo, spongy parenchyma; st, stomata; x, xylem.

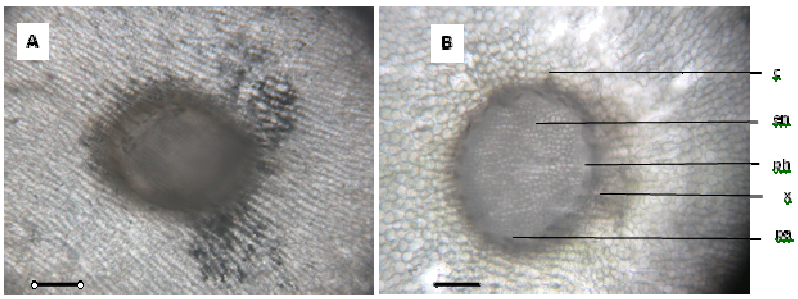


Fig. 7. Transverse section of vitro root oil palm untreated control A: treatment with WPM containing 7.2 % sucrose supplemented with 6 mg NAA and 9 mg PBZ B: Note that difference in thickness are largely attributable to size of cells. Abbreviations: c, cortex; en, endodermis; ph, phloem; x, xylem; pa, stellar parenchyma.

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