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Optimal medium for watercress (*Alternanthera* sp.) micropropagation

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Abstract

The objective of the present study was to find the optimal conditions for micropropagation of *Alternanthera* sp. The first experiment aimed to find the optimal sterilization of lateral bud meristems for *in vitro* culture. The results revealed that the best sterilization technique is to clean lateral bud meristem by bending shoot and dipping in 70% ethanol for 1 min. These shoots were then removed and immersed in 3% sodium hypochlorite solution for 10 min. This sterilization technique provided the highest survival percentage of 95% and the best microorganism removal. The second experiment aimed to determine the effects of the plant growth regulators benzyladenine (BA) on shoot proliferation. The shoot induction of sterile lateral bud meristems of *Alternanthera* sp. was then carried out in Murashige and Skoog (MS) media supplemented with benzyladenine (BA) at five different concentrations (0, 1, 2, 3 and 4 mg/l) under 25±2 °C and light condition for 4 weeks. It was found that MS media supplemented with 3 mg/l BA induced the optimal growth and development of shoots. This condition provided the highest root induction and the significant average shoot of 12.88±0.51 shoots/explant ($p<0.05$). The results from the present study could be the basic information for producing aquatic plants that have economic value and genetic conservation. In additions, it may be useful for biological activity tests or provide the possibility for callus induction for further somatic embryo culture technology.

Keywords: *Alternanthera* sp., Micropropagation, Sodium hypochlorite, Benzyladenine

1. Introduction

Watercress is a favorite vegetable as salad and other menu among people in Europe and some countries in Asia. There are two species of watercress grown in Thailand. The first one is *Nasturtium officinale* (family Brassicaceae), which is an imported species from Europe. It contains isothiocyanate which exhibits anti-cancer activity (Kopsell, Barickman, Sams, & McElroy, 2007).

Another watercress species that is widely grown in Thailand is *Alternanthera* sp. (family Amaranthaceae). Its commercial name is Asian watercress or Japanese watercress. It is a herbaceous plant, commonly found in freshwater pond and swamp. Watercress varieties might be imported, mutated or genetically modified to have different appearances. Phytochemical study revealed that watercress (*Alternanthera* sp.) comprises various bioactive compounds including phenolic compounds, alkaloids and flavonoids (Majumder, Rashid, Chowdhury, Gupta, & Mandal, 2016). The stem and leaf of watercress highly compose of ferric, vitamin A, proteins and fibers (Dutta, 2015). In folklore medicine, watercress is used as anti-pyretic and anti-ulcer agent (Rattanathongkom, Sripanidkulchai, & Kanchanapoom, 2008).

In additions, it is used for treatment of diarrhea, common cold and gastro-intestinal disorders (Kumar, Dheeba, Stalin, Maragatham, & Kannan, 2011; Nin, 1986). It is reported that watercress inhibits the growth of pathogenic virus in respiratory tracts, dengue virus (Jiang, Yang, Chen, Xiao, & Luo, 2007) and human immunodeficiency virus (HIV) (Zhang, He, Tabba, & Smith, 1988). Watercress has virus inhibitory activity. It may play a significant role on immune system stimulation (Rattanathongkom, Sripanidkulchai, & Kanchanapoom, 2008).

The present study was aimed to investigate the optimal conditions for micropropagation of watercress

(*Alternanthera* sp.) which is widely grown in Thailand. This is the basic information for producing aquatic plants that have economic value using tissue culture for consumption and genetic conservation. In addition, this may be useful for biological activity tests and for pharmaceutical purposes. Moreover, it could provide the possibility for callus induction for further gene transfer study by somatic embryo culture technology. Therefore, the objective of this study is to investigate the optimal sterilization technique for micropropagation and study the effect of BA on lateral bud meristems in *Alternanthera* sp.

2. Materials and Methods

2.1. The optimal sterilization technique for micropropagation

Mature watercress without diseases and pests were cleaned with tap water. There are two protocols for the investigation of the optimal sterilization technique for micropropagation of lateral bud meristems in *Alternanthera* sp., as follows;

(1) The lateral bud meristems with length 1-1.5 cm were cleaned with tap water and liquid soap. These lateral buds were then compared in the sterilization technique by putting in sodium hypochlorite at the concentration of 10 and 15 % for 10 and 20 min. The specimens were then washed with sterile distilled water for 2 minutes, 3 times. The exceeded lateral buds were removed, then the optimal sterilization technique for micropropagation was determined by culture on MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2.5 g/l agar (Phytagel®) and pH 5.7 for 4 weeks. The experimental design was completely randomized design (CRD) with 24 replications. The results were recorded in terms of contamination and survival of the explants within 1 week (figure 1).

(2) Watercress lateral bud meristems 1-1.5 cm in length were soaked in 70% ethanol for 1 min and left at room temperature for 15 min until dry. The shoots were cut for comparison of the sterilization technique with sodium hypochlorite at the concentration of 0, 1, 2 and 3 % for 10 min. The specimens were then washed with sterile distilled water for 3 times. The exceeded lateral buds were removed. The explants were then cultured on MS media (Murashige and Skoog, 1962) supplemented with 3% sucrose, 2.5 g/l agar (Phytagel®) and pH 5.7 for 4 weeks. The experimental design was CRD with 24 replications. The results were recorded in terms of contamination and survival of the explants within 1 week (figure 1).

2.2. The effects of BA on shoot induction from lateral bud meristems

Watercress lateral bud meristems were sterilized from experiment 1. The exceeded parts were removed by cutting leaves. The shoots meristems were cut to -1.5 cm in length. They were then cultured on MS media supplemented with benzyladenine (BA) at different concentrations of 0, 1, 2, 3 and 4 mg/l, 3% sucrose, 2.5 g/l agar (Phytagel®) and pH 5.7 under temperature of 25±2°C for 4 weeks and sub cultured every two weeks. The growth parameters of the explants including shoot length, shoot counts and morphology of the explants were recorded. The experimental design was CRD with 15 replications. The statistical differences among means were compared by using Duncan's new multiple range test (DMRT). The confidence interval was 95% ($p<0.05$).

3. Results

3.1. The optimal sterilization technique for micropropagation

Watercress lateral buds were compared for the sterilization technique with sodium hypochlorite at the concentration of 10 and 15 % for 10 and 20 min. The results revealed that lateral buds sterilized with sodium hypochlorite at the concentration of 10 and 15 % for 10 min had the percentage of bacterial and fungal contamination of 100 %. Lateral buds sterilized with sodium hypochlorite at the concentration of 10 % and shaken for 20 min had the percentage of bacterial and fungal contamination of 54.16 % and maximum survival rate of 45.83 %. Interestingly, lateral buds sterilized with sodium hypochlorite at the concentration of 15 % and shaken for 20 min had the percentage of bacterial and fungal contamination of 29.16 % and maximum survival rate of 70.83 % (Table 1).

Next, were the results for the method in which lateral bud meristems were soaked in 70% ethanol followed by sterilization with sodium hypochlorite at concentrations of 0, 0.5, 1, 2 and 3 % for 10 min. It was found that watercress shoots sterilized with 70% ethanol in combination with 3% sodium hypochlorite had the lowest microbial contamination percentage of 5 %, followed by shoots sterilized with 70% ethanol in combination with 2% sodium hypochlorite with a microbial contamination percentage of 21 %. For shoots sterilized with 70% ethanol in combination with 1% and 0.5% sodium hypochlorite, these had a contamination percentage of 100 % without any survival. The sterile explants were effectively induced as new shoots in the next experiments (figure 2).

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Table 1. Contamination and survival rates of lateral bud meristems after sterilization with different treatments on MS media

Treatments	Chemicals and sterilization methods	Means (%)	
		Contamination rate	Survival rate
1	10% NaOCl, 10 min	100	0
2	10% NaOCl, 20 min	54.16	45.83
3	15% NaOCl, 10 min	100	0
4	15% NaOCl, 20 min	29.16	70.83
5	70% EtOH + 0.5% NaOCl	100	0
6	70% EtOH + 1.0% NaOCl	100	0
7	70% EtOH + 2.0% NaOCl	21	79
8	70% EtOH + 3.0% NaOCl	5	95

Figure 1. Characteristics of *Alternanthera* sp., A) before sterilization; B) after sterilization; C) lateral buds after exceeded part removal

Figure 2. Microbial contamination on MS media, A) bacterial contamination; B) fungal contamination; C) sterile and surviving lateral buds

3.2. Effects of BA on watercress lateral bud meristematic tissues for 4 weeks

Watercress lateral buds were cultured on MS media supplemented with benzyladenine (BA) at different concentrations of 0, 1, 2, 3 and 4 mg/l under temperature of 25±2 °C and light condition in order to induce shoots for 4 weeks. The results showed that lateral buds cultured on MS media supplemented with BA at different concentrations had significant different shoot counts and lengths ($p<0.05$). MS media supplemented with 4 mg/l BA induced the highest average shoot count of 13.66±1.86 shoots/explant, followed by MS media supplemented with 3, 2 and 1 mg/l BA which induced the shoot count of 12.88±0.51, 12.22±1.01 and 11.87±1.05 shoots/explant, respectively. The MS media without supplementation of BA induced the lowest shoot count of 4.44±0.84 shoots/explant (Table 2 and Figure 3).

The study on the effects of BA on the shoot lengths revealed that MS media without supplementation of BA induced the highest average shoot lengths of 3.37±0.32 cm, followed by MS media supplemented with 1, 2, 3 and 4 mg/l BA which induced the shoot lengths of 2.50±0.23, 2.42±0.23, 2.37±0.32 and 2.35±0.10 cm, respectively. MS media supplemented with 4 mg/l BA induced the lowest average shoot length of 2.35±0.10 cm. The MS media without BA had a significant number of induced shoot length. Whereas, explants cultured on MS media supplemented with BA at different concentrations did not have significant different shoot lengths when the means were compared by using DMRT at confidence interval of 95% (Table 2).

The observation of morphological changes of lateral bud meristematic tissues showed that MS media supplemented with 4 mg/l BA induced a lot of new shoots. When cultured for several weeks, leaves did not expand but were clustered. They were elongated with a yellowish green color and the root induction was very few. However, MS media supplemented with 3 mg/l BA induced the better explants. Leaves were completely green in color. The root induction was complete with more root counts (data not shown). Therefore, MS media supplemented with 3 mg/l BA is recommended for further micropropagation of *Alternanthera* sp.

Table 2. The effects of different concentrations of BA supplemented in MS media on shoot counts and shoot lengths in watercress lateral bud meristematic tissues cultured for 4 weeks

Media formula	The growth of watercress lateral bud meristems*	
	Average shoot counts (shoots/explant)	Average shoot lengths (cm)
MS only	4.44^{a}±0.84	3.37^{b}±0.32
MS supplemented with 1 mg/l BA	11.87^{b}±1.05	2.50^{a}±0.23
MS supplemented with 2 mg/L BA	12.22^{b}±1.01	2.42^{a}±0.23
MS supplemented with 3 mg/l BA	12.88^{b}±0.51	2.37^{a}±0.32
MS supplemented with 4 mg/l BA	13.66^{b}±1.86	2.35^{a}±0.10

Remarks: * is means from 3 replications

Different letters after numbers in the same column mean there is significant difference compared by DMRT at confidence interval of 95%

Data are expressed as mean±S.D. (standard deviation)

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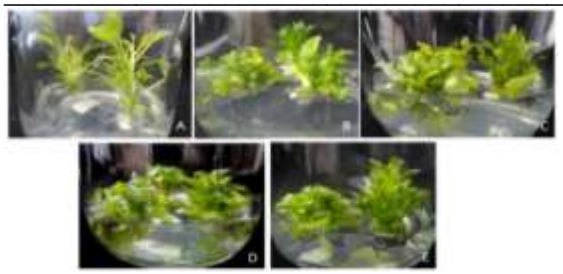

Figure 3. The growth of watercress lateral bud meristematic tissues cultured on MS media only and MS media supplemented with BA at four different concentrations for 4 weeks. A) MS only; B) MS + 1 mg/l BA; C) MS + 2 mg/l BA; D) MS + 3 mg/l BA; E) MS + 4 mg/l BA

4. Discussion

Disinfection is an important step in the tissue culture process. The most commonly used screening method is sodium hypochlorite solution, which has capacity in inhibiting microorganisms on the surface of tissues. The appropriate concentration of bleach depend on the type of plant and the parts to be sterilized. According to Rawdkhao (2000), plant tissues should be subjected to the removal of microorganisms on the surface using sodium hypochlorite solution in various concentration. The optimal concentration of sterilizing agents that provide the most sterile plant tissues should be further investigated. These sterilizing agents must be easily eradicated and not harmful to plant tissues.

In the present study, MS media (Murashige & Skoog, 1962) was selected in the investigation of optimal concentrations of BA in the micropropagation of watercress lateral bud meristematic tissues. MS media is a general media formula for plant tissue cultures. It comprises of various kinds of nutrients (Tanthai et al., 2015). MS media is usually supplemented with BA at different concentrations. It was found that all concentrations of BA affect the growth of watercress lateral bud meristematic tissues. BA belongs to cytokinin plant regulator. It stimulates and accelerates somatic cell division, cell expansion and differentiation of lateral buds into shoots in a very short time (Sakakibara, 2006; Zhang, Swarup, Bennett, Schaller, & Kieber, 2013). The previous studies showed that MS media supplemented with cytokinin plant regulators increases the shoot counts in micropropagation of the genus *Alternanthera* and other aquatic plants. Shekhawat, Manokari and Revathi (2017) reported that MS media supplemented with 2 mg/l BA induced a lot of shoot counts (23.8±1.9 shoots/explant) in micropropagation of *Alternanthera philoxeroides*. Flores, Flôres, Bempck, Maldaner and Marchioretto (2016) found that MS media supplemented with 1 µM Thidiazuron (TDZ) in combination with 30 or 40 g/l sucrose and 20 g/l glucose effectively increases the shoot counts in micropropagation of *Alternanthera hirtula*. Pongchawee, Pasugdee, Pradissan, Pipatcharoenchai and Kanthrong (2012) reported that MS media supplemented with 2 mg/l BA significantly increases induction of meristematic tissues of *Echinodorus osiris* Rataj into shoots ($P<0.05$) with average shoot counts of 3.31±0.74 shoots/explant, average root counts of 4.81±1.17 roots/explant, average shoot height of 2.45±0.16 cm and average leaf counts of 7.75±0.82 leaves/explant. In addition, it was found that MS media supplemented with 1 mg/l BA significantly increases shoot counts ($P<0.05$) of water trumpet (*Cryptocoryne tonkinensis*) in comparison to the other concentrations with average shoot counts of 4.50±2.12 shoots/explant (Pongchawee, Chusang, & Tong-mee-aied, 1999).

In the present study, although MS media supplemented with 4 mg/l BA induced the higher average shoot counts than those of 3 mg/l BA, leaf growth in watercress is incomplete. This finding may be due to the increase of BA concentrations which will be toxic to meristematic tissues and therefore exhibiting plant growth inhibition. In additions, Montri et al. (2000) found that MS media supplemented with 10 mg/L BA decreases shoot counts in the micropropagation of lateral bud meristems of *Phyllanthus emblica*. Six weeks after culture of watercress lateral bud meristematic tissue, it was found that the shoots were very small in size. Leaf growth was incomplete. Root counts were quite low. These findings probably related to the high accumulation of BA in MS media in the early phase of the experiment. When the concentrations of BA were still high, there were changes in the balance of plant growth regulators in plant tissues resulting in the incomplete growth of plants (Pikulthong, Teerakathiti, Thamchaipenet, & Peyachoknagul, 2016). Likewise, Lee and Chan (2004) reported a stunted shoot of *Orthosiphon stamineus* developed in the medium with increasing concentrations of BA. However, MS media without supplementation of plant growth regulators induced the lowest average shoot counts. The shoots were elongated. Leaves were largely expanded. Root counts were very high. Cytokinin has an important role on transportation of nutrients including vitamins and minerals in plants. Therefore, meristematic tissues cultured on MS media without supplementation of BA had the decreased shoot counts.

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5. Conclusion

The most suitable method for surface sterilization in *Alternanthera* sp. lateral bud meristematic tissues was the direct bending of shoot and exposure to 70% ethanol for 1 min and left at room temperature until dry. The shoots were then cut and soaked in 3% sodium hypochlorite for 10 minutes. This technique provided the contamination rate of 5% with the highest survival rate of 95%. It should be recommended for further micropropagation of lateral bud meristematic tissues of *Alternanthera* sp.

The optimal conditions for the culture of the aquatic plant *Alternanthera* sp. using tissue culture were through the use of MS medium containing 3 mg/L BA. The plant grown for a further one week could develop new shoots. The results from the present study could be applied for micropropagation of lateral bud meristematic tissues for other aquatic plants and biological activity tests as well as secondary metabolite production for pharmaceutical purposes. In addition, it could provide the possibility for callus induction for further gene transfer study by somatic embryo culture technology.

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