

EFFECT OF PLANT PRESERVATIVE MIXTURE PPMTM ON THE SHOOT REGENERATION OF WATERCRESS (*NASTURTIIUM OFFICINALE*)

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ABSTRACT:

Plant Preservative Mixture™ (PPM), a relatively new broad-spectrum preservative and biocide for use in plant tissue culture, was evaluated as an alternative to the use of conventional antibiotics and fungicides in plant tissue culture. Culture inoculated in MS media supplemented with PPM (1.5 ml/l) was the effective concentration which gave the best values. The top values were recorded for all studied characters using apical buds compared with lateral buds. The combination between apical buds and (1.5 ml/l) PPM concentration showed the superior values of all studied parameters (33.25%, 19.40%, 14.46%, 20.53% and 79.46%) (60.55%, 39.80%, 20.97%, 45.33% and 54.44%) and (31.20%, 20.06%, 12.33%, 35.13% and 81.06%) for contamination, % bacterial contamination, % offungi contamination, dead explants% and survival explants% respectively. Different concentrations of PPM (0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml/l) were tested using single node and apex explants of watercress (*Nasturtium officinale*). PPM at (1.5ml/l) had significant effect on the studied characters; shoots height, shoots number, leaf pairs number, fresh and dry weight which they reaches (4.16, 4.62, 42.00, 0.524 and 0.063 g, respectively). Apex bud explants showed the greatest effect on shoots height shoots number, leaf pairs number, fresh and dry weight and their values were 3.52, 32.02, 3.38, 0.405 and 0.036 g, respectively. The best parameter were recorded on MS media supplemented with PPM at (1.5ml/l) with apex buds explant (4.55, 5.34, 46.28, 0.570 and 0.085, respectively) for shoots height, shoots number, leaf pair number, fresh and dry weight. Current study aimed to determine the best concentration of PPM for limiting the contamination of watercress and micro shoot regeneration.

KEYWORDS: *Nasturtium officinale*, PPM™, Contamination, Antibiotic.

1. INTRODUCTION

Watercress (*Nasturtium officinale* R.Br.) is a perennial plant, which is native to Eurasia and naturalized throughout North America. It grows submerged, floating on water, or spread over mud surfaces in cool, flowing streams. White flowers are followed by small, beanlike seedpods. *N. officinale*, which contains a glucosinolate named gluconasturtiin, has been traditionally used for treatment of diabetes, an endocrinal chronic disease caused by altered carbohydrate metabolism and characterized by elevated blood glucose levels. *N. officinale* extracts showed an antioxidant activity via reducing cellular lipid peroxidation, reducing power, free radical and superoxide anion radical scavenging activities (Bahramikia and Raziheh 2010; Mazandarani *et al* 2012 and Boligon *et al* 2013)

Plant tissue culture involves the culture of all types of plant cells, tissues and organs under aseptic conditions. This definition also extends to the culture of excised embryos to protoplast culture. The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of season and weather (Smith, 2000). The use of tissue culture technique for the vegetative propagation of plants is the most widely used application of the technology. It has been used with all classes of plants (Murashige 1978; Conner and Thomas, 1981).

Microbial contamination is the single most important reason for explant loss in plant tissue culture and one of the most

serious problem of plant tissue culture laboratory (Boxus and Terzi, 1987; Cassells, 1991; Leifert *et al.*, 1994). Plant Preservative Mixture™ (PPM) (Plant Cell Technology, Washington, D.C.) is a relatively new, broad-spectrum preservative and biocide for use in plant tissue culture. The active ingredients are 5-chloro-2-methyl-3(2*H*)-isothiazolone and 2-methyl-3(2*H*)-isothiazolone. PPM is effective against both bacteria and fungi, heat stable and unlike conventional antibiotics, can be autoclaved in the media. These characteristics of PPM make it an attractive alternative to using conventional antibiotics and fungicides in plant tissue culture.

The literature survey revealed that no work has been done to propagate the watercress (*Nasturtium officinale* L.) By plant tissue culture in Kurdistan region. Thereby, this study was conducted to propagate and decrease the bacterial contamination of *Nasturtium officinale* by using PPM™.

2. MATERIALS AND METHODS

This investigation was conducted at tissue culture laboratory, College of Agriculture, University of Duhok during the period from February 1st to May 1st 2014. The medium solidified with agar was used in *invitro* growth experiments (Murashige and Skoog, 1962) premixed medium purchased from Caisson Company USA. Watercress plants were collected from fields in Zawa and Kanimasea villages in Duhok city, Kurdistan Region of IRAQ. Plants were transferred under greenhouse conditions in the College of Agriculture, University of Duhok, grown at containers, containing mixture of sandy loam soil (2:1 v/v). Apex

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and lateral buds from greenhouse grown plants of watercress were used for explants preparation. Stems (apex buds) and lateral buds were removed from their main axis and washed with tap water for 60 minutes to remove soil and other superficial contaminates followed by tap water and detergent as liquid soap for 20 min, followed by 3 – 5 min rinses in sterile distilled water, then surface sterilization of shoot tips and single nodes was done by immersing them in a flask containing 100 ml 0.1% mercuric chloride (V/V) for 7 min with one drop of wetting agent (Tween-20) to enhance the sterilization. Then, the apex and lateral buds were rinsed 3 times using sterilized distilled water. All the tissues visibly damaged by the sterilizing were removed. The stems were cut into suitable explants as apex bud into (1.0 cm) long and lateral bud were cut into (1.0 cm) long.

Two explants were cultured in jars containing 25 ml solidified MS media and 10 replications were used for each treatment. The cultures were maintained at $25 \pm 1^\circ\text{C}$ and $60 \pm 5\%$ relative humidity in a culture room under 16 h photoperiod provided by white fluorescent tubes (light intensity 1000 lux). The records on % survival of explants were made after 2 weeks of incubation.

2.1 Antibacterial Treatments

Contamination difficulties were faced during undertakes study. In this respect, many trials had been followed to avoid contamination through the use of different antibiotics. This is a summary to the trials that were carried out on the bacteria *Pseudomonas aerudomonasa* which was diagnosed by bacteriology laboratory at the university.

Table 1. Effect of different PPMTM concentrations, explant Bacterial, Fungi, Dead explants and survival percentage using both apical and lateral buds

Explant types	PPM (ml/l)						Mean of explant type
	explant contamination %						
	0.0	0.5	1.0	1.5	2.0	2.5	
Lateral buds	98.83 a	85.00 c	70.33 e	35.30 k	44.76 i	55.20 i	64.90 a
Apical buds	94.83 b	78.96 d	66.80 f	31.20 l	41.63 j	49.86 h	60.55 a
Mean of PPM	96.83 a	81.98 b	68.56 c	33.25 f	43.20 e	52.53 d	
Explant type	Bacterial contamination %						Mean of explant type
Lateral buds	81.40 a	54.06 c	34.73 e	18.73 k	23.13 h	32.33 f	40.75 a
Apical buds	78.30 b	52.96 d	53.10 e	20.06 j	21.90 i	30.50 g	39.80 b
Mean of PPM	79.85 a	53.51 b	34.91 c	19.40 f	22.51 e	31.41 d	
Explant type	Fungi contamination %						Mean of explant type
Lateral buds	17.86 f	30.93 b	36.10 a	16.60 g	21.63 d	22.86 d	24.33 a
Apical buds	16.53 g	25.63 c	31.83 b	12.33 h	19.73 e	19.73 e	20.97 b
Mean of PPM	17.20 d	28.28 b	33.96 a	14.46 e	20.68 c	21.31 c	
Explant type	Dead explants%						Mean of explant type
Lateral buds	89.13 a	62.70 c	39.06 g	22.13 J	38.66 g	49.00 e	50.11 a
Apical buds	84.56 b	60.83 d	31.70 i	18.93 K	35.13 h	40.83 f	45.33 B
Mean of PPM	86.85 a	61.76 b	35.38 e	20.53 F	36.90 d	44.91 c	
Explant type	Survival%						Mean of explant type
Lateral buds	11.13 j	37.30 h	61.03 e	77.86 B	62.33 e	49.60 f	46.87 b
Apical buds	15.43 i	39.16 g	68.30 c	81.06 A	64.73 d	60.96 e	54.94 a
Mean of PPM	13.28 e	38.23 d	64.66 b	79.46 A	63.53 b	55.28 c	

*Means followed by the same letter for each factor and interaction do not differ significantly from each other's according to Duncan's Multiple range Test at 5% level.

2.2 Experiments

Tow experiments were carried out to exam the effect of ppmtm. The first experiment was conducted to evaluate the effect of multiple concentrations and combinations of PPMTM on explants contamination. PPMTM was purchased from Caisson Company, USA, characterized by its broad-spectrum preservative and biocide, which kills bacteria and fungi cells, prevents germination of spores, and in higher

concentration, can eliminate explants of endogenous contamination. The explants were cultured in jars containing 25 ml solidified MS media supplemented with PPM at (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5ml/l) based on recommended doses (plant cell technology, 1998) supplemented with BA at (1.5 mg/l). Tow explants were cultured in each jar and 10 replicates were used for each treatment. After 4 weeks of incubation, contamination, bacterial contamination, fungi contamination, Dead explants and % survival explants percentage (%) were recorded.

The second experiment was conducted to study the effect of different concentrations and combinations of PPMTM on *in vitro* propagated. The explants were cultured in jars containing 25 ml solidified MS media supplemented with PPM at (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5ml/l) combined with 1.5 mg l⁻¹ BA. Two explants were cultured in each jar and 10 replicates were used for each treatment. After 4 weeks of incubation, mean shoot length, number of shoots, number of leaf pairs and fresh and dry weight of explant were recorded.

All cultures were re-cultured every two weeks, because of rapid growth of plantlet; as a result of it is fast-growing plant. The cultures were maintained at the same conditions of previous experiment.

2.3 Data Analysis

The experiments were designed according to Complete Randomized Design (CRD) using 5 replicates for each treatment. Data were analysed and means were compared with using Duncan's multiple range tests at 0.05 levels. Data scored on % were subjected to arcsine transformation before statistical analysis and then converted back to % for presentation. All statistical analysis was performed using the computerized program of SAS (SAS, 2007).

3. RESULTS AND DISCUSSION

The visual observation showed very early contamination in explant cultures within the first days. More than half of the cultures became rapidly contaminated. The results in Table (1) indicated that supply PPM on the MS media revealed significant effect on all studied characteristics especially at (1.5 ml/l) concentration, where the values were (33.25%, 19.40%, 14.46%, 20.53% and 79.46%) for explants contamination%, bacterial contamination%, fungi contamination%, dead explants % and % of survival percentage respectively. On the other hand, Table (1) explains that there were significant differences when apical bud cultured on the MS media compared with lateral bud. Apical buds recorded the highest value of the for explants contamination%, bacterial contamination%, fungi contamination %, dead explants % and % of survival percentage respectively which they were (60.55%, 39.80%, 20.97%, 45.33% and 54.44%).

Table 1 illustrates the clear effects of the interaction between PPM concentration and the explant type on the studied parameters. The best results were achieved when apical bud cultured on MS media supplemented with (1.5 ml/l) and they were (31.20%, 20.06%, 12.33%, 35.13% and 81.06%) for contamination, bacterial contamination, fungi contamination, dead explants and survival. These results agreed with those of Niedz (1998) who explained the effect of PPM on several types of propagated citrus that were by tissue culture on reducing bacterial and fungi contaminations. Fuller and Pizzey (2001) investigated the important role of PPM to limited the contamination of brassicas culture media when it used at (1.5ml/l) concentration. Neidz and Michael (2002) reported that the bud explant of *Citrus sinensis* L. grown on culture media containing (1.5ml/l) PPM was routinely controlled the contamination for (95% clean). Also, Jimenez and Jhman (2006) mentioned that PPM at (1.5 to 2 ml /l) effectively reduced contamination of the explant of *Guadua angustifolia* after explant inoculation on media. According to

Rihan *et al.*, (2012) who noticed that the cauliflower seeds were cultured on media supplied with (1.0ml/L⁻¹) was most effective elimination on bacterial contamination in culture system. This corresponds with Babaei *et al.*, (2013) who indicated that the PPM at both concentrations (1 and 2 ml/l) eliminated the microbial contamination of Lemba (*Curculigolatifolia*) explants.

This study confirmed the positive effect of PPMTM in eliminating the contamination and highlighting the value of using PPM which was refer to be species-depending since the use of PPM comparatively at high concentration could have negative effect on the development of plant tissue. The explant is predominant essential source of contamination and there are microorganisms on surface live loping of buds and outer layers (Miyazaki *et al.*, 2011). Additionally, many explants could be suitable harbor internal microbial contamination, could have contamination within vascular system and intercellular spaces in leaf mesophyll, xylem vessel (Donnarumma *et al.*, 2011). Also, there is a general acceptance to Bunn and Tan proposal (2002) who proposed that plant materials from field is generally more contaminated compared to the plants grown in greenhouses. Babeie *et al.*, (2013) confirmed that the plants that grown in high level of humidity contain more sources of contaminate compared to that grown in dryer environment.

Worth to mention that the used watercress explants in the current study were collected from the field seven though they were kept in the greenhouse and as is known that watercress originally is a herbaceous aquatic plant, which was extremely challenging to disinfected and the common protocols for explant sterilizing could be less effective because of having a heavy load of external and internal contamination sources. When the explants inoculated in the media, the competition will happened between microbial and plant material on media nutrients, whereas bacterial contamination often need growth factors which are represented in tissue culture media.

Disinfestation using PPMTM contaminates on watercress bud explant were eliminate due to PPM role in preventing the germination of both bacteria and fungi spores and the inhibition of airborne contamination, waterborne contamination and contamination caused by operator contact as well as in many cases it is used to reduce the endogenous contamination (Plant cell technology, 2003).

Results in Table 2 show that PPM had the greatest effect on explants regeneration. As appeared from the current data the highest length of shoots increased significantly by increasing PPM concentration till (1.5 ml/l) and it was (4.16 cm) then decreased by increasing PPM concentration. On the other hand the shoots length varies according to explant type that the apical buds 3.38 cm exceed the lateral buds 2.73cm.

The interaction effects also appeared significant differences, the PPM at (1.5 ml/l) with apical bud gave the highest average of explant height (4.55 cm). Table 2 shows differences among different concentrations of PPM. The results showed that number of shoot /explant was significantly increased by PPM, the maximum shoot number /explant (4.62 shoot) was obtained when explants were cultured on MS media supplemented with (1.5 ml/l). Apical buds had the highest rate of number of shoots /explant (3.52 shoot) compared with lateral buds.

Significant increase in the average number of shoots /explant was noticed by interaction between PPM concentration and explant part. The highest number of shoots /explant (3.52 shoot) was obtained in media supplied with (1.5 ml/l) of PPM. PPM concentrations significantly affect the pair leaves

number/explant which (42.00 leaf pairs) at (1.5 ml/l). Concerning the number the results also show increasing leaves pair numbers using apical buds (32.02) at the same time the media supplemented with (1.5 ml/l) reaches the highest numbers of leaves pair (46.28).

Table 2. Effect of different concentrations of PPMTM on highest shoot length, shoots number, leaf pairs, fresh and dry weight using apical and lateral buds.

Explant type	PPM (ml/L ⁻¹)							Mean of explant type	
	0.0	0.5	1.0	2	2.5	1.5	2.0		2.5
Lateral buds	1.75 j	2.73 g		3.56 e		3.78 d	2.68 G	1.89 i	2.73 b
Apical buds	2.70 g	3.86 c		4.32 b		4.55 a	2.82 F	2.03 h	3.38 a
Mean of PPM	2.22 e	3.29 c		3.94 b		4.16 a	2.75 D	1.69 f	
Explant type	Average of shoots number/explant							Mean of explant type	
Lateral buds	1.50 e	2.50 d		3.90 c		3.90 c	2.44 D	1.50 e	2.62 b
Apical buds	2.44 d	4.64 b		4.56 b		5.34 a	2.46 D	1.68 e	3.52 a
Mean of PPM	1.97 e	3.57 c		4.23 b		4.62 a	2.45 D	1.59 f	
Explant type	Average of leaf pairs number/explant							Mean of explant type	
Lateral buds	17.44 i	31.32 e		35.60 d		37.72 c	25.28 G	18.20 h	27.59 b
Apical buds	16.16 j	38.06 c		44.32 b		46.28 a	28.78 F	18.52 h	32.02 a
Mean of PPM	16.80 f	34.69 c		39.69 b		42.00 a	27.03 D	18.80 f	
Explant type	Average of fresh weight/explant							Mean of explant type	
Lateral buds	0.265 j	0.378 f		0.426 e		0.479 c	0.347 G	0.224 k	0.353 b
Apical buds	0.332 h	0.460 d		0.502 b		0.570 a	0.307 I	0.257 j	0.405 a
Mean of PPM	0.298 e	0.419 c		0.464 b		0.524 a	0.327 D	0.240 f	
Explant type	Average of dry weight/explant							Mean of explant type	
Lateral buds	0.017 c	0.018 c		0.028 c		0.041 b	0.021 C	0.016 c	0.023 b
Apical buds	0.021 c	0.033 b		0.039 b		0.085 a	0.022 C	0.019 c	0.036 a
Mean of PPM	0.019c	0.026 b		0.034 b		0.063 a	0.021 c	0.017 c	

*Means followed by the same letter for each factor and interaction do not differ significantly from each other's according to Duncan's Multiple range Test at 5% level.

Data presented in Table 2 indicated that a significant increase in the average of fresh weight (0.524 g) was recorded when (1.5 ml/l) of PPM was added to the MS media. Also, significant increase (0.405 g) of fresh weight was observed when apical buds were cultured on the media. The highest rate of fresh weight (0.570 g) was reached via adding 1.5 ml/l PPM using apical buds. Table 2 clarifies the significant effect of PPM at (1.5 ml/l) concentration on the average of dry weight /explant (0.063 g). Results clearly indicated that significant differences appeared between the apical buds and lateral buds and the highest value (0.036 g) recording using apical buds. The variation in dry weight rate was observed during the experiment when PPM at (1.5 ml/l) concentration added to the media with culturing apical buds and it was (0.085 g).

These results agreed with those reported by George and Robert (2001) who mentioned that PPM at (0.5 and 1.0 ml/l) had a positive effect on the average of explant forming shoots per explant in birch and rhododendron, but dramatically had no significant effect on chrysanthemum. These results are supported by the recommendation of Plant Cell Technology

(1998) that PPM decreased or reduced air born contamination in media.

Compton and Jennifer (2001) suggested that the suitable concentration of PPM should be less than (2ml/l), which can be safely and effectively used for adventitious organ regeneration of petunia, tobacco and melon. Also, Rihanet *et al.*, (2012) observed that the addition of PPM on culture media had a positive effect on the average number of developing micro shoots and average of weight using (1ml/l). According to Babaeet *et al.*, (2013) who referred that the survival average of Lemba (*Curculigolatifolia*) shoot tip explant was significantly high regeneration when it cultured on media containing (1 and 2 ml/l). Therefore, PPM concentration range is necessary to be examined when it used for first time on our locality.

Contamination often minimized the accuracy of plant cells and tissue culture system even if it exists in small amounts in the cultural environment where it effects in temperature or pH or other components can be pronounced. Immediate reproduction of microorganism and contamination can lessen explants regeneration and growth or kill the plant cells and tissues in plant micro propagation (Cooke *et al.*, 1992; Lifert and

waites,1992).Contamination losses during *in vitro* stages of plant tissue culture could ultimately minimized by plant preservative mixture PPM™ (Leifert and Cassells, 2001). It was postulated that plant growth regulators were used primarily for plant material growth and development and the explant cannot develop in plant growth regulator free tissue culture media in partnership with proper concentration of PPM (Rihanet *et al.*, 2012).

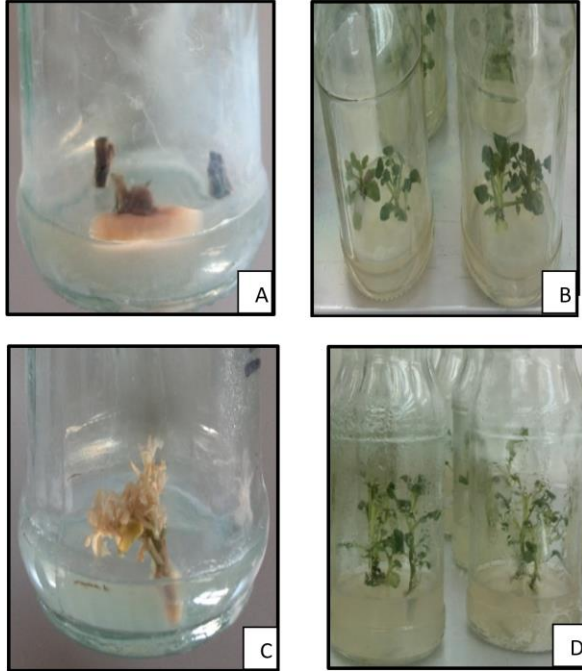


Figure 1. Effect of PPM at 1.5 mg/l on lateral and apical explant regeneration. A: lateral bud explant cultured on MS media at 0.0ml/l PPM. B: lateral bud explant cultured on MS media supplemented with 1.5ml/l PPM after four weeks in culture. C: Apical bud explant cultured on MS media at 0.0ml/l PPM. D: Apical bud explant cultured on MS media supplemented with 1.5ml/l PPM after 4 weeks in culture

In this study, the presence of PPM in culture media with BA create convenient environmental culture for watercress explant regeneration, where PPM eliminated the contamination and BA, stimulates apex bud development (De sliva *et al.*,2003). The reasons that BA, might be has internal molecular structure and number of double bonds on this chain of benzyl ring, it may increase the acting of plant growth regular than other cytokine (Hopkins and Hiiner, 2004). Additionally, BA is most effective cytokinins in cell division compared with other cytokinins (Al-Ansary *et al.*, 2007).

PPM controls microbes by penetrating the microbial cell wall and inhibiting several key enzymes in the citric acid cycle and electron transport chain (Plant Cell Technology, 1998). It may also inhibit transport of monosaccharides and amino acids from the media into the microbial cells. The complexity of the plant cell wall apparently prevents the PPM molecules from having the same effect on plant tissues (Plant Cell Technology, 1998). However, PPM's effect on metabolic or transport pathways apparently can also affect the ability of leaf explants from certain species to form adventitious shoots as demonstrated by the significant increase in the regenerative ability of watercress explants. Therefore, the effects of PPM on explants regeneration should be tested before using it in experiments or commercial production. The results of a current study concluded that the preservative

plant mixture PPM™ can be used effectively to control the growth of fungi and bacteria in plant tissue culture.

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