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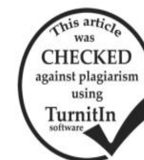
Agrobacterium Rhizogenes and B12 Antioxidant Mediated Rooting Induction and Shoots Proliferation on Micro-Cuttings of Pyrus Betulaefolia Rootstock



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ABSTRACT

The present research carried out to compare in vitro rooting as well as shoot proliferation and elongation of Pyrus betulaefolia shoots after infecting basal part with Agrobacterium rhizogenes suspension strain DSM 30200 and vitamin B_{12} at either 1.0 or 1.5 mg/L and two combinations between them. Untreated control micro-cuttings failed completely to induce adventitious roots. Otherwise, Agrobacterium rhizogenes inoculation treatment gave the best in vitro effect on rooting performance and root parameters of the treated micro-cuttings. Addition of antioxidant (vitamin B_{12}) at 1.0 and 1.5 mg/L to culture medium showed positive effects on rooting formation and root parameters with special emphasis to the concentration of 1.5 mg/L. This later treatment, among the examined ones, recorded the earliest start rooting (10.33 days) and the highest value of explants survival and rooting percentages (50 and 50.33%, respectively). As for shoots proliferation and elongation parameters, it showed the better response than the applied 1.0 mg/L "T_2" in most measured parameters and it occupied the next rank after A. rhizogenes inoculation treatment "T_1" in that respect. In contrast, the combined treatments failed completely to induce adventitious roots and recorded the lowest levels of shoot multiplication and elongation parameters on the treated micro-cuttings.

Keywords: Pyrus betulaefolia, Hypocotyls, Agrobacterium rhizogenes, Micro-cuttings, Vitamin B_{12}, rooting performance, Adventitious root formation

INTRODUCTION

Pyrus betulaefolia is commonly known for Japanese pears cultivations. It was used as rootstock in the last few years in a commercial scale because it has several advantages (Hassanen and Gabr, 2012). Multi-purpose of P. betulaefolia rootstock has recently reported, it has been selected for tolerates growth under saline conditions as it possesses higher tolerance to draught (Stebbins, 1995). This rootstock is one of the best rootstocks which is tolerant to wet and draught conditions, resistance to pear decline, fire blight, root aphid and root rot. However, it is often difficult to root which is still the major obstacle to successful micro-propagation of this important rootstock Paul and silver (2002).

Plant growth promoting rhizobacteria (PGPR) have recently been used to stimulate roots formation by secretion of plant growth promoting substances (Burdman *et al.* 1997).In the same line, McAfee *et al.* (1993) attributed rooting on the transformed cells in cuttings after infection to an increase in cellular auxin sensitivity rather than auxin production. Such activating mechanism has already proven by Haggman and Aronen, (2000) who stated that A. rhizogenes, known for root formation in carrot disks and apple trees may induce adventitious rooting in woody plants by incorporating the rolB gene and/or by secretion of compounds that stimulate rooting. It was also found that it increased rooting of Prosopis chilensis in tissue culture systems.

Vitamin B_{12} (antioxidant) works in close participation with A. rhizogenes in the current study based on the main function of antioxidants in protecting cells from oxidative injury throughout scavenging the created free radicals (Award *et al*., 2001 and Kondo *et al*., 2005). Likewise, it is important as a co-factor for characterization of certain biological substances lead to create thyamidylic acid and purine nucleotide precursor of nucleic acids (DNA and RNA) synthesis which is necessary for normal cell division process. Both also are involved in the synthesis of proteins as activator of amino acids as well as carbohydrates and fats metabolism (Cannon *et al*., 2002). In addition, vitamin B_{12} function is important to provide micro-cutting with sufficient food (Carbohydrates) needed for root development and bud sprouting (Hartmann *et al.* 2011).

The present work is an attempt to study the possibility to overcome the difficult to root problem on micro-cuttings of Pyrus betulaefolia rootstock using infection with Agrobacterium rhizogenes cells and antioxidant vitamin B_{12} added to culture medium at 1.0 and 1.5 mg/L either solely or in combinations.

MATERIALS AND METHODS

The present experiment occurred in Tissue Culture Laboratory and Greenhouse of Horticulture Department, Faculty of Agriculture, the University of Mansoura, EL-Mansoura, Egypt. Seeds were extracted from ripe fruits on pear (Pyrus betulofolia L.) trees apparently healthy grown in a private orchard in 6th October district. Such seeds were washed carefully under running water and mixed with 3 drops of tween 20 with shaking for 30 min followed by washing again with running water. Seeds were sterilized by immersing into absolute ethanol for one minute followed by rinsing in sterile water then soaked in 50% commercial Clorox solution (5% sodium hypochlorite) for 20 min to eliminate seed borne diseases. To avoid any Clorox residual on seeds, they were rinsed four times in renewed sterile distilled water under a laminar air flow hood.

Seeds were cultured in sterilized Petri dishes (10 cm), 20 seeds each contained sterilized double filter papers and kept in refrigerator at 4°C for 30 days to overcome embryo dormancy condition. The seeded Petri dishes were transported to plant growth rooms at 25°C till seeds germinated. Uniform plantlets (in vitro grown) at 40-day-old of about 2 cm long hypocotyls and one milliliter diameter were selected to be source of explants each of 1.5 cm in long (micro-cuttings). Scalpel blade and forceps were used for preparation of micro-cuttings.

Bacterial strain and cultivation

Agrobacterium rhizogenes strain DSM30200 sourced from Cairo Mircen Laboratory was examined in this experiment. The bacterial cells were aerobically cultured on Luria Bertani (LB) medium composed of

tryptone (10.0 g/L), yeast extract (5.0g/L), Na CL (10.0g/L), agar (15.0 g/L) and at pH 7 (Draper *et al.* ,1988). Bacterial cell were inoculated in LB medium and left 3 days at 27±1°C. Transformation was done as a single colony used to inoculate 5 ml of LB liquid medium and grow overnight at the same temperature under continuous shaking at 120 rpm. One milliliter of overnight grown Agrobacterium culture was used to inoculate 25 ml of LB medium at 27±1°C, shaken till an optical density 0.6 at 600 nm wave length, followed by centrifugation under cooling at 4200 rpm for 20 min. The supernatant was discarded and Agrobacterium cells precipitate pallet was suspended in 25 ml of MS (1962) liquid basal medium.

Agrobacterium rhizogenes inoculation procedure

Inoculation process was carried out according to the technique described by Damiano *et al*. (1995). The basal part (5.0 mm) of fresh wounded micro-cuttings was dipped in 0.5 ml bacterial suspension for 24 hrs in complete darkness. Before the beginning of cultivation process on MS (1962) basal medium, the excess of bacterial cells suspension on the inoculated micro-cuttings was absorbed through sterile filter papers.

Micro-cuttings culturing

Infected basal part of micro-cuttings were cultured for 48 hrs on MS (1962) basal medium hormones and antibiotics-free in the presence or absent of vitamin B_{12} (Cyanocoblamin) antioxidant then re-cultured on the same medium containing the antibiotic Cefotaxime (500 mg/L) to inhibit further bacteria growth. Culturing process was done in complete darkness for 10 days, however the upper parts were exposed to white light (1500 lux) for 16 hrs photoperiod provided by white fluorescent tubes at 27±1°C. To avoid the side effect of applied antibiotic in the medium, three days later the tested micro-cuttings transported and re-cultured on the same medium antibiotic-free.

Tested treatments

Infection with Agrobacterium rhizogenes cells and antioxidant vitamin B_{12} were added to culture medium at 1.0 and 1.5 mg/L either solely or in combinations. Five treatments plus uninfected control micro-cuttings were used to examine their effect on adventitious roots formation and shoots proliferation parameters on the treated micro-cuttings.

Evaluation parameters

The tested treatments were evaluated throughout measurement of physical parameters measured on the treated and cultured micro-cuttings of pear rootstock at 5-week-old .These parameters included: percent survival, leaf number, shoot number, shoot length, percent rooting, root number, root length, root diameter were measured. In addition, the plantlets were washed with distilled water and the fresh and dry weight of the shoots and roots were calculated

Experiment design and statistical analysis

Experiment was designed in a randomized complete block design with 6 treatments on the tested micro cuttings, 3 replicates with 10 cultured tubes each. This means 30 micro-cuttings per treatment.The obtained data were subjected to analysis of variance (ANOVA) by using "Genstat 11.1" (2008). The mean comparisons were performed by the least significant difference value (LSD) at 5% level of probability according to the method described by Gomez and Gomez. (1984).

RESULTS AND DISCUSSION

Rooting performance and adventitious root parameters

Effect of the tested treatments on rooting performance and adventitious root parameters, measured on the treated micro-cuttings, was indicated from the concerned data in Table (1) and illustrated in Figure (1). From these data, it was cleared that untreated control micro-cuttings on MS basal medium "Tc" completely failed to induce adventitious roots. However, these micro-cuttings produced shoots measured the lowest levels of proliferation parameters (Table 2 and Figure 1). These findings confirmed that rooting of Pyrus betulaefolia micro-cuttings in vitro has proven difficult.

The difficult rooting on pear cuttings was recorded since early times by Fadle and Hartmann, (1967) and Fadle, (1967). They reported that Bartlett pear hardwood cuttings are very difficult to root under treatment and it is related distinctly to the presence of high amounts of inhibitors corresponding with promoters. The easy rooted cuttings had strong promoters, while the difficult to root ones possessed strong inhibitors. In addition, they indicated that these inhibitors were produced in the buds and interfered with metabolic reactions that lead to adventitious root production. The present result also in complete agreement with those indicated in the studies of Bhojwani *et al*. (1984), who compared the ability to induce roots on cuttings of both pear scions and rootstocks and found that scion cultivars have proved more difficult to root than rootstocks. DePaoli, (1989) and Reed, (1995) with Pyrus spp. also go to similar results. The same was true with results in the studies of Stimart and Harbage, (1989), who failed to induce rooting in micro-cuttings of P. calleryana cv. Bradford even after 2 years of subculture and intermittent attempts at rooting. In the same line, Zhu *et al.* (2001) used Agrobacterium tumefaciens in order to improve rooting ability in pear (Purus communis, L.) rootstock through transformed this rootstock by the rolB gene isolated from A. rhizogenes. In vitro rooting results showed that the transgenic clones rooted from 67 to 100 % without auxin, while the untransformed control did not root at all on the hormone free rooting medium.

The previous table and figure proved also micro-cuttings when pre-culturing infected with A. rhizogenes cells without the addition of vitamin B_{12} in MS basal culture medium "T_1" succeeded to early start rooting (12 days) and tabulated significantly the highest average value of rooting parameters compared with the other tested treatments. This means that the best in vitro response to induce adventitious roots on Pyrus betulaefolia shoots was when infected basal part with A. rhizogenes cells before culturing on MS basal medium. Similar results were found for the effect of infection with these bacterial cells on shoot proliferation and elongation parameters, since the highest level of such parameters was measured on micro-cuttings under this super treatment "T_1" (Table 2 and Figure 1). Rooting performance and adventitious roots formation by infecting the basal part of P. betulaefolia micro-cuttings

with A. rhizogenes bacterial cells are due to integration and subsequent expression of bacterial DNA apportion (T-DNA) from the root inducing (Ri) plasmid in the plant genome. This root induction in woody plants was formed by incorporating the rolB gene and /or secretion of compounds that stimulate rooting McAfee *et al*. (1993). Our results agreed also with those resulted in the studies of McAfee *et al*. (1993) who worked on rooting of Pinus monticola, Pinus banksiana and Larix laricina micro-cuttings. In the same line, Dobigny *et al*. (1995) demonstrated that hairy roots on micro-cutting of two potato cultivars were induced after inoculation with two strains of A. rhizogenes. Aronen *et al*. (1996) with Pinus sylvestris reported that A. rhizogenes significantly stimulated rooting without genetically transforming the plants and would alleviate the concerns over release of genetically modified organisms with the beneficial of increased rooting. Similar results were reported by Caro *et al*. (2003) with P. chilensis micro-cuttings cultured in tissue culture system after infected with A. rhizogenes cells. More recent, Felker *et al*. (2005) examined effect of four strains of A. rhizogenes on improving the rooting percentage of recently identified clones of Prosopis Alba (algarroba bloco). They found that this bacterium has a stimulate effect on rooting of difficult to root woody species. Recently, Abou Rayya *et al*. (2010) came also to similar results on bitter almond micro-cuttings. Infected ones before culturing succeeded to induce rooting (95.00 – 99.00 %), while those uninfected and grown under open field failed completely to root.

Results of the present study indicated also that the next response on inducing adventitious roots on Pyrus betulaefolia micro-cuttings was to the treatments of supplemented culture medium with antioxidant vitamin B_{12} either at 1.0 mg/L "T_2" or 1.5 mg/L "T_3". The later one was the superior among the examined treatments and recorded significantly the earliest start rooting (10.33 days) and the highest micro-cuttings survival and rooting percentages of 50.00 and 40.33 %, respectively (Table 1 and Figure 1). As for other measured root parameters, it was the next after "T_1" treatment to measure the average values for roots number, roots length, roots diameter, roots fresh weight and roots dry weight per micro-cutting (Table 2 and Figures 1). While it gave the highest value of roots number (3.33) as compared with other treatments. Additive of antioxidant to culture medium has been considered also as effective in improving shoot proliferation and elongation parameters. Once more, the added concentration of 1.5 mg /L "T_3" was the more effective, since it the measured higher average values per micro-cutting for most shoot proliferation parameters if compared with the same measures on micro-cuttings under "T_2" treatment (Table 2).

The above positive effect of the applied antioxidant vitamin B_{12} with special emphasis to 1.5 mg/L concentration "T_3" to rank the next increasing effect on rooting ability of Pyrus betulaefolia micro-cuttings is based on the active role of vitamin B_{12} in combination with other medium constituents to induce direct or indirect effects on callus growth, somatic growth and rooting. The direct effect is through increasing formation of thyamidylic acid and purine nucleotides procure to synthesis of the building block of nucleic acids (DNA and RNA). Both are involved in synthesis of proteins as for as carbohydrates and fats metabolism which they all are necessary for normal cell division (Cannon *et al*. 2002).

Table 1. Effect of Agrobacterium inoculation and vitamin B_{12} antioxidant (VB_{12}) in culture medium either solely or in combinations on Rooting parameters

Treatments	Start of rooting (days)	Micro-cuttings survival (%)	Micro-cuttings rooting(%)	Root number	Root length (cm)	Root diameter (mm)	Roots fresh weight (mg)	Roots dry weight (mg)
Tc. Control	0.00	40.00	0.00	0.00	0.00	0.00	0.00	0.00
T_1 A. rhizogenes inoculation	12.00	45.00	42.67	1.67	13.67	0.83	15.50	2.28
T2VB_{12} at 1.0 mg/L	11.33	50.00	40.33	1.33	4.10	0.80	9.20	1.36
T3 VB_{12} at 1.5 mg/L	10.33	50.00	40.33	3.33	4.60	0.80	12.00	1.82
T4 A. rhizogenes + VB_{12} at 1.0	0.00	35.00	0.00	0.00	0.00	0.00	0.00	0.00
T5 A. rhizogenes + VB_{12} at 1.5	0.00	35.00	0.00	0.00	0.00	0.00	0.00	0.00
New LSD at 0.5%	0.93	0.94	0.73	0.73	1.11	0.04	0.16	0.02

As for the negative effect of the combined treatments, between A. rhizogenes cells and vitamin B_{12} at the two tested concentrations "T_4&T_5", on rooting performance and adventitious root parameters was very similar to that resulted in untreated control micro-cuttings "Tc". Micro-cuttings under these treatments failed completely to induce adventitious roots on the treated micro-cuttings (Tables 1 and Figures 1). Likewise, the lowest response to special emphasis to "T_4" treatment on shoot proliferation parameters was observed. These negative responses of the tested combined treatments, in spite of their component factors solely appeared an active effect, may be due to some bad interactions between bacterial cells and antioxidant vitamin B_{12} were possibly happened. The presence of a high amount of inhibitors corresponding promoters plus other specific chemical and biochemical cellular components in basal section of Pyrus betulaefolia difficult to root micro-cuttings may be played a positive role activates these bad interactions . Consequently, an inhibiting effect on A. rhizogenes cells activity as well as vitamin B_{12} function to induce adventitious roots and minimizing most shoot proliferation and elongation parameters on the treated micro-cuttings could be happened. Original evidence supported this explanation came from observation on effect of buds and leaves on rooting of cuttings, since certain inhibitors such as phenols have been found in buds regardless of phase of adventitious root formation. Such inhibitors appear to differ significantly between difficult and easy to root cuttings (Hartmann *et al*., 2011).

Tc T1 T2 T3 T4 T5

Figure 1. Photo showing the effect of Agrobacterium rhizogenes inoculation and vitamin B_{12} antioxidant in MS culture medium .

The above suggested causatives of the inferior effect of the combined treatments in the present study are in harmony with the results of Damiano nd Monticelli, (1998) study which aimed to compare rooting on micro-cuttings of different genotypes related to almond, apple, plum and two hybrids rootstocks. The obtained findings indicated that rooting percentages tended to decrease with the combination treatments between auxin and pre-culture A. rhizogenes inoculation. Future researches should be aimed to throw future lights identified factors that play a role on the presence of negative effects on adventitious root formation and shoot proliferation observed on micro-cuttings under the combined treatments examined in the current investigation. The obtained results could be explained a new approach to an additional modern technique that can be helps in overcoming the difficulty to root micro-cuttings. Furthermore, this characterized technique provides uniform rootstock seedlings true to type that insure complete similar effects on the grafted or budded scions. In addition, it improves performance of the induced adventitious roots as well as increases rooting % and shoot proliferation parameters in order to achieve a successful transfer to the field. These benefits beside the accepted advantages of plant tissue culture, as the main instrument for plant biotechnology, make Pyrus betulaefolia plantlets produced can be considered good rootstock if the propagated trees are desirable.

Table 2. Effect of Agrobacterium inoculation and vitamin B_{12} antioxidant (VB_{12}) in culture medium either solely or in combinations on shoot proliferation parameters

Treatments	Symbol	Shoots number	Shoots length (cm)	Shoot diameters (mm)	Leaves number	Shoots fresh weight (mg)	Shoots dry weight (mg)
Control	Tc	1.00	3.10	0.90	10.33	39.13	5.87
A. rhizogenes inoculation	T_1	1.00	7.20	1.13	16.33	138.50	20.78
VB12 at 1.0 mg/L	T_2	1.00	3.50	1.07	10.33	88.80	17.20
VB12 at 1.5 mg/L	T_3	1.00	5.13	1.17	11.67	174.80	24.48
A. rhizogenes + VB12 at 1.0	T_4	1.00	2.20	1.03	9.33	110.13	14.66
A. rhizogenes + VB12 at 1.5	T_5	1.00	5.20	1.07	11.00	112.73	18.04
New LSD at 0.5%		*	0.17	0.09	1.19	0.15	0.02

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توسط أجروباكتريوم ريزوجينيس ومضاد الأكسدة فى استحداث التجذير ونمو الأفرخ على العقل الدقيقة لأصل كمثرى البيتيولوفوليا

لطيف جرجس سمعان ، محمود ابراهيم القاضى ، أمير محمد ناجي شعلان و لمياء محمود محمد محمود
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أجريت الدراسة فى معمل زراعة الأنسجة لقسم البساتين بكلية الزراعة جامعة المنصورة على عقل دقيقة من أصل كمثرى البيتيولوفوليا الناتجة عن زراعة بذور استخلصت من ثمار ناضجة جمعت من أشجار كمثرى بيتيولوفوليا سليمة ظاهريا نامية فى حديقة خاصة فى منطقة 6 أكتوبر. تعرضت العقل المختبرة قبل الزراعة فى بيئة موراشيج و سكوج الأساسية (MS 1962 basal medium) لستة معاملات واحدة عدوى الجزء القاعدى من العقل بخلايا بكتريا الأجروباكتيريوم ريزوجينيس Agrobacterium rhizogenes عن طريق النقع لمدة 24 ساعة فى معلق الخلايا ومعاملتين بزراعة العقل فى نفس البيئة مع تزويدها بمضاد أكسدة فيتامين ب$_{12}$ عند تركيزين 1.0، 1.5 ملجم/لتر والجمع بينهما فى معاملتين آخرتين أما السادسة فهى المعاملة المقارنة(الكنترول) وفيها زرعت العقل الدقيقة بدون أى معاملة فى نفس بيئة الزراعة الأساسية وتحت نفس الظروف. أظهرت النتائج المتحصل عليها بعد خمسة أسابيع من تاريخ الزراعة ما يلى : فشلت تماما العقل الدقيقة للمعاملة المقارنة (الكنترول) فى استحداث التجذير بينما انتجت أفرخ سجلت أقل مستويات التضاعف والاستطالة بالمقارنة مع المعاملات الأخرى. أظهرت معاملة عدوى قواعد العقل الدقيقة بخلايا البكتريا ثم الزراعة فى بيئة الزراعة الأساسية بدون أى اضافات نجاح ملحوظ فى الوصول الى بداية تجذير سريعة وتسجيل أعلى متوسط قيم على الصفات الجذرية والخضرية المقاسة على العقل الدقيقة المعاملة بالمقارنة مع مثيلاتها تحت المعاملات الأخرى. سجلت النتائج الخاصة بمعاملتى اضافة مضاد الأكسدة بتركيزين لبيئة الزراعة وخصوصا عند تركيز1.5ملجم/لتر أعلى متوسط قيم للصفات الجذرية المختبرة كما برهنت أنها أكثر فعالية لتحسين تضاعف واستطالة الأفرخ الناتجة على العقل الدقيقة المعاملة بالمقارنة مع نتائج المعاملة الأخرى بتركيز 1.0 ملجم/لتر. فشلت تماما العقل الدقيقة تحت المعاملتين المختلطتين بين العدوى بالخلايا البكتيرية واضافة مضاد الأكسدة لبيئة الزراعة فى استحداث التجذير على العقل الدقيقة المعاملة كما سجلت قيم فقيرة لصفات تضاعف واستطالة الفروخ الناتجة بذلك تشبه تماما التأثير السلبى المدون لعقل المعاملة المقارنة.