Adv. Hort. Sci., 2020 34(1): 43-48 DOI: 10.13128/ahsc-8300

AHS
Advances in Horticultural Science

Cefixime manages internal bacterial contamination during tissue culture operation

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Key words: antibiotic, *in vitro* culture, growth, *Pyrus communis.*

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Citation:
AMERI A., DAVARYNEJAD G.H., TEHRANIFAR A., MOSHTAGHI N., 2020 - *Cefixime manages internal bacterial contamination during tissue culture operation* - Adv. Hort. Sci., 34(1): 43-48

Data Availability Statement:
All relevant data are within the paper and its Supporting Information files.

Competing Interests:
The authors declare no competing interests.

Received for publication 20 October 2018
Accepted for publication 6 March 2019

Abstract: Large-scale propagation of *Pyrus communis*, which is a difficult-to-root species, is dependent on tissue culture technique. However, internal bacterial contaminations are an obstacle in tissue culture of fruit tree species. For this purpose, this investigation was conducted with several experiments to manage bacterial contamination. First, gram test for bacterial contamination related to *Pyrus* shoots proliferating was conducted. Gram test revealed that bacteria contaminating pear shoots were negative gram. Then, we investigated the application of cefixime (0, 100, 300 and 500 mg L^{-1}) or ampicillin (0, 100, 300 and 500 mg L^{-1}) for managing bacterial contaminations. It was found that the contaminated area on medium containing 500 mg L^{-1} cefixime (63.585 mm^2) was lower than other treatments (803.84 mm^2). Therefore, cefixime at 500 mg L^{-1} was selected to control the bacterial contamination. Next, different procedures were used included shaking with (1: sterile distilled water, 2: 500 mg L^{-1} cefixime and culturing in media with 500 mg L^{-1} cefixime, 3: 500 mg L^{-1} cefixime, culturing and subculturing in media with 500 mg L^{-1} cefixime 4: Disinfection). The third procedure was known the best due to the low bacterial contamination percentage and rate also the healthy growth of plants. Finally, the effect of gibberellic acid at 0 and 1 mg L^{-1} was investigated to compensate for shoot growth reducing in the presence of cefixime. 1 mg L^{-1} gibberellic acid improved the growth indices in the presence of cefixime.

1. Introduction

Fire blight, the most devastating disease of pear, leads to the death of the whole pear tree through the systematical infection in all underground and aerial parts of the tree (Vanneste, 2000; Evrenosoğlu *et al.,* 2019). From the horticultural science perspective, the revival of the pear orchards is dependent on large-scale propagation. The majority of cultivated pear is *Pyrus communis* (Morgan *et al.,* 1994) and *P. communis* cv. Williams is sensitive to fire blight (Abdollahi *et al.,* 2010). However, *Pyrus communis* is difficult-to-root (Zhu *et al.,* 2003; Sun *et al.,* 2011); therefore, the tissue-culture technique can support large-scale propagation of pear.

One of the most important factors in tissue culture is the control of microbial contamination. Plant immune system acts against pathogens. There are three steps of plant defense responses included the response in the step of entry, establishment, and spread of pathogens. Overall, plant immune systems can be classified into cell wall reinforcement and programmed cell death. In pathogen entry condition, cell wall reinforcement is efficient and in pathogen establishment and spread conditions programmed cell death can be restrictive (Abramovitch and Martin, 2004). When pathogens suppress the plant defenses responses, the plant was invaded by pathogens and contaminated. One of the phytopathogens is bacteria; they cause serious troubles *in vitro* conditions. Some of the modes of bacteria actions to suppress the host defenses refer to the use of type III effector proteins and toxins (Abramovitch and Martin, 2004) as well as, type IV secretion systems to inject effector proteins into cells (Angot *et al.,* 2007). Tissue culture technique is very sensitive to special pathogenic factors and all of the microbes in air condition and equipment. In other words, all of the microbes infect cultures with the aim of nutrition (Nadha *et al.,* 2012). Internal infections in plant cultures had often harmful effects for shoot proliferation, shoot rooting and quality of plant growth (Nadha *et al.*, 2012). In tissue culture, the selection of the appropriate antibiotic is important. There are several reports about the use of antibiotics to manage the bacterial contamination (Phillips *et al.,* 1981; Falkiner, 1990; Kneifel and Leonhardt, 1992; Barrett and Cassells, 1994; Falkiner, 1997; Nadha *et al.,* 2012). Cefixime is an antibiotic belonging to cephalosporin class. The mode of action of cephalosporins is related to inhibiting the cell wall biosynthesis so that this class arrests the formation of peptide bonds (Kohanski *et al.,* 2010).

Affect sites of various antibiotics are different; therefore the efficiency of different antibiotics is different in the removal of bacteria. There are some observations for different antibiotics affect sites in the previous studies, such as, inhibiting of cell wall synthesis that is related to benzylpenicillin and phosphomycin, inhibiting protein synthesis related to chloramphenicol and streptomycin, inhibiting of RNA and DNA synthesis related to rifampicin and nalidixic acid (Phillips *et al.,* 1981). One of the side effects of antibiotic application in plant tissue culture is the reduction of growth. However, in control of bacterial infection in *Guadua angustifolia*, streptomycin sulfate decreased growth shoot, but kanamycin caused intensive growth with high-quality; therefore, the effects of antibiotics are different on plant growth (Nadha *et al.,* 2012). The management of contamination in tissue culture leads to the prevention of waste of time and energy. In this investigation, we examine the use of antibiotics to manage the bacterial contaminations coupled with the use of GA_3 to compensate for the poor growth of the plant in the presence of antibiotic in the media.

2. Materials and Methods

Plant materials

Three months old proliferating micro-shoots of *Pyrus communis* cv. Williams, as the most common cultivar in the world, exhibited bacterial contamination. Contaminated micro-shoots were picked to investigate the experiments of bacterial control for the large-scale production of *P. communis*. These micro-shoots were maintained in MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 3% sucrose.

Gram test of bacteria

Two drops, approximately 50 µL, of a 3% (W/V) solution of potassium hydroxide were placed on a clean glass slide as outlined by Ryu (1940). Bacterial cells were transferred from culture media aseptically with a flat wooden toothpick and placed into the drop of KOH with rapid, circular agitation. After 5-8 sec, the toothpick was alternately raised and lowered just off the slid surface to detect a stringing effect. It was considered gram-negative bacteria if drop viscosity increased within 15 sec (Suslow *et al.,* 1982; Schaad *et al.,* 2001).

Antibiotic selection test

Contaminated shoots were cultured on MS medium (Murashige and Skoog, 1962) supplemented with either ampicillin 0, 100, 300 and 500 mg L^{-1} or cefixime at 0, 100, 300 and 500 mg L^{-1}. Vessel cultures with 32 mm inner diameter were maintained at a constant temperature of 25±1°C and in 16/8 h light/dark photoperiod (45 µmol m^{-2} s^{-1}) using cool white fluorescent lamps (Sylvania, Germany). After a week, the contaminated area was measured in each treatment.

Bacterial contamination removing

In the before step, we selected the proper antibiotic (cefixime at 500 mg L^{-1}). Then, we used four different procedures using cefixime at 500 mg L^{-1} to control any eventual bacterial contamination. For each procedure, 3 micro-shoots were cultured in a

candle jar as a replicate.

1) Shaking of contaminated shoots with sterile distilled water (control);

2) The first, shaking of contaminated shoots with cefixime at 500 mg L^{-1} then, cultured in media with cefixime at 500 mg L^{-1}. Finally, sub-culturing in free antibiotic media;

3) The first, shaking of contaminated shoots with cefixime at 500 mg L^{-1}, then, cultured in media with cefixime at 500 mg L^{-1}. Finally, sub-culturing in media with cefixime at 500 mg L^{-1};

4) Disinfection of contaminated shoots (immersing in 1% hypochlorite sodium for 5 min then rinsed with sterile water three times).

MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 3% sucrose were used for each procedure. Cultures were maintained at a constant temperature of 25±1°C and in 16/8 h light/dark photoperiod (45 µmol m^{-2} s^{-1}) using cool white fluorescent lamps (Sylvania, Germany). After 30 days, several traits were evaluated: percentage of fungal contamination and bacterial contamination (BC), bacterial contamination rate (BCR) and general health.

Based on the following equation (E1) bacterial contamination rate was counted per each micro-shoot in each candle jar:

$$BCR = \sum \frac{Ni}{Di}$$

where BCR= Bacterial contamination rate, N_i = Number of the contaminated shoot in each day, D_i = Day number.

Rescued shoot improvement

Micro-shoots related to the best procedure were transferred to MS medium (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 3% sucrose, 500 mg L^{-1} cefixime and gibberellic acid (GA_3) treatments. The concentrations of GA_3 were 0 and 1 mg L^{-1}. pH was adjusted at 5.8 with NaOH prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 0.8% agar. Cefixime antibiotic and GA_3 added to the media after autoclaving by filtering. Related traits of this experiment were included: the percentage of new growth, the percentage of proliferation, the average number of bud and leaf, as well as, the average shoot length.

The evaluation of declined antibiotic dose

After the six months using cefixime at 500 mg L^{-1}, proliferated shoots were divided into two groups. Each group of plants was cultured in media with cefixime at either 500 mg L^{-1} or 250 mg L^{-1}. pH was adjusted at 5.8 with NaOH prior to autoclaving at 98 kPa and 121°C, and the media were solidified using 0.8% agar. Cefixime antibiotic and GA_3 were added to the media after autoclaving with the syringe filter (pore size: 0.22 µm). After 10 days, the percentage of bacterial contaminations and bacterial contamination rate were measured per each micro-shoot in each candle jar based on (E1).

Rooting micro-shoots

After six months, micro-shoots were transferred to ½-strength QL (Quoirin and Lepoivre, 1977) medium supplemented with 1.5 mg L^{-1} Naphthaleneacetic acid (NAA) and 500 mg L^{-1} cefixime. Cultures were maintained a week in dark conditions, then, transferred to 16/8 h (light/dark) photoperiod and light intensity of approximately 45 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) emitted by cool-white fluorescent tubes in 35% relative humidity.

Statistical analysis

These experiments were arranged as a completely randomized design with three replications. The data were statistically analyzed using a one way ANOVA test and means were compared with the Duncan test at the 5% level of confidence. All of the statistical tests were performed using SAS (Statistical Analysis System) software v9.1. All of the percentage data were transformed to Arcsin √x.

3. Results and Discussion

The result of the gram type detection showed contamination of this investigation is related to gram-negative bacteria. In the antibiotic selection step, we compared ampicillin and cefixime. We used 0, 100, 300 and 500 mg L^{-1}concentrations of each antibiotic. Ampicillin is a common antibiotic in tissue culture, and its activity spectrum is related to gram-positive and gram-negative bacteria, whereas the cefixime is antibiotic acting against gram-negative bacteria. In antibiotic selection test, results revealed 500 mg L^{-1}cefixime could overcome contamination better than other treatments. The contaminated area on medium containing 500 mg L^{-1}cefixime (63.585 mm^2) was lower than other treatments (803.84 mm^2). Therefore, we selected 500 mg L^{-1} cefixime to control the contamination for later experiments.

ANOVA revealed that the difference between the four procedures was significant ($P \leq 0.01$) for bacterial contamination percentage and the rate of bacterial

contamination (Table 1). Four procedures were used for the survival and rescuing of shoot from bacterial contamination. The third procedure (the first, shaking of contaminated shoots with 500 mg L^{-1} cefixime; then, cultured in media with 500 mg L^{-1} cefixime. Finally, sub-culturing in media with 500 mg L^{-1} cefixime) was known the best due to the low bacterial contamination percentage, rate and finally the healthy and fresh growth of plants. These indices in other procedures were not desirable; as the highest percentage of bacterial contamination was observed in the first procedure (shaking with sterile distilled water). All of the procedures led to necrotic plants except for procedure 3 (Table 2). The presence of antibiotic in media effected on plant growth and weakened their growth; therefore, we used GA_3 and its effect evaluated on plants growth. GA_3 application in media containing 500 mg L^{-1} cefixime had a significant effect on the percentage of new growth, the average of bud number, the average shoot length and the average of leaf number ($P<0.05$) (Table 3). Without the application of GA_3 were not observed any proliferation and new bud formation; while in media containing GA_3, 22.53% proliferation and 1.46 the average number of bud were observed (Table 4). As well as, the results showed the decrease in the antibiotic dose to 250 mg L^{-1} cefixime increased BCP

Table 1 - Analysis of variance traits under study

Source of variance	DF	Means square	
		Bacterial contamination	Rate of bacterial contamination
Treatment	3	2963.807 **	45.48649 **
Error	8	7.350.308	0.5436

NS= no significant; **= significant at the 0.01 level of probability according to Duncan Test.

Table 2 - Evaluation of different procedure to come over bacterial contamination during the culture

Procedure	Fungal contamination (%)	Bacterial contamination (%)	Bacterial contamination rate	General health after 30 days
1	0	90 ± 0	9.47 ± 0.77	Necrotic leaves
2	0	74.55 ± 2.42	4.93 ± 0.119	Necrotic leaves
3	0	17.01 ± 1.22	0.45 ± 0.053	Green leaves and healthy
4	0	57.85 ± 1.56	7.47 ± 0.323	Necrotic leaves

1= shaking with sterile water.
2= the first, shaking of contaminated shoots with cefixime (500 mg/l) then, culturing in media with antibiotic. Finally, sub-culturing in free antibiotic media.
3= the first, shaking of contaminated shoots with cefixime (500 mg/l), then, culturing in media with antibiotic. Finally, sub-culturing in with antibiotic media.
4= disinfection (immersing in 1% hypochlorite sodium for 5 min then shaking with sterile water for three times.
Values are mean ± standard error.

Table 3 - Analysis of variance of traits under study

Source of variance	DF	Means square				
		Percentage of new growth	Percentage of proliferation	Average of bud number	Average shoot height	Average of leaf number
Treatment	1	514.20 **	761.53 **	3.22 **	60.16 *	54.0 *
Error	4	6.022	4.53	0.0066	7.33	0.33

**= significant at the 0.01 level of probability according to Duncan Test.
*= significant at the 0.05 level of probability according to Duncan Test.

Table 4 - Evaluation of Gibberellic acid in MS medium along with PGRs and 500 mg/L cefixime on secondary growth traits

Concentration (mg/L)	New growth (%)	Proliferation (%)	Average bud number	Average shoot height (mm)	Average leaf number
1	43.93 ± 1.23	22.53 ± 1.74	1.46 ± 0.07	9.66 ± 2.03	10.33 ± 0.33
0	25.41 ± 1.58	0 ± 0	0 ± 0	3.33 ± 0.88	4.20 ± 0.33

Values are mean ± standard error.

to 41% and BCR to 1.2 (Table 5). Therefore, using the antibiotics at 500 mg L^{-1} should continue because the plants grow without bacterial contamination only in the presence of 500 mg L^{-1} cefixime (Fig. 1). Nadha *et al.* (2012) stated the removal of kanamycin from the medium did not result in resumption contamination after 10 days (Nadha *et al.,* 2012); while other literature mentioned that the usage of antibiotics for inhibiting the bacteria growth has impermanent impact and removal of antibiotics has accompanied by resumption contamination (Falkiner, 1990; Barrett and Cassells, 1994; Falkiner, 1997; Leifert and Cassells, 2001) confirming the results of this experiment. In the consumption of antibiotic, resistant-bacteria theory is undeniable. Despite long-term using of cefixime, about six months, it could not only remove bacterial contamination, but also act without any resistant-bacteria. Finally, rescued shoots were able to produce healthy roots.

Table 5 - Evaluation of decreasing of cefixime on BCR and BCP after ten days

Traits	Cefixime concentration (mg/L)	
	250	500
Bacterial contamination rate	41 a	0 b
Bacterial contamination percentage	1.2 a	0 b

Different letters in columns indicate significant difference between treatments at 5% level.

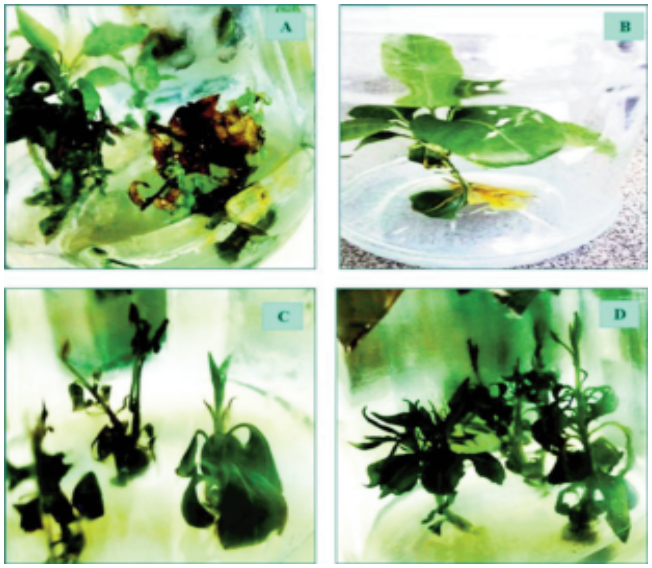

Fig. 1 - Contaminated shoots *in vitro* culture (A). Contaminated shoot after sub-culturing on media without cefixime (B). New growth after using GA3 treatment (C, D)

Leifert and Cassells (2001) mentioned alternatives for the antibiotic in their review. These alternatives included medium acidification and autotrophic culture (e.g. culture without carbohydrate) (Leifert and Cassells, 2001). In other literature were noted to activating of endogenous bacteria as a result of sub-culturing in media with cytokinins (Kneifel and Leonhardt, 1992). However, plant tissue culture without cytokinins, carbohydrates and with the modification in media acidity is impossible. In this regards, this investigation showed with the presence of cefixime in media containing cytokinins, carbohydrates could manage the bacterial contamination. Based on the results of this investigation, cefixime at 500 mg L^{-1} had not any toxicity effect on growth and proliferation. Cefixime is an antibiotic belonging to cephalosporins class. The cephalosporin antibiotics have been introduced as the appropriate antibiotic plant tissue culture since they have low eukaryote toxicity (Mathias and Boyd, 1986) which our results emphasize this point.

4. Conclusions

Bacterial contamination incidence is common and unavoidable during the in vitro propagation of fruit tree species. This investigation presented a procedure to manage the bacterial contamination of *P. communis* cv. Williams during the *in vitro* culture. Based on the results of this investigation, cefixime at 500 mg L^{-1} could control the bacterial contamination. The use of antibiotic in a medium is associated with a decrease in the growth of plants. This side effect of antibiotic was managed with the application of GA_3 at 1 mg L^{-1}. Therefore, we suggest cefixime at 500 mg L^{-1} for *in vitro* propagation of fruit trees.

References

ABDOLLAHI H., TAHZIBI F., GHAHREMANI Z., 2010 - *Correlation between fire blight resistance and morphological characteristics of pear (*Pyrus communis *L.).* - Acta Horticulturae, 896: 339-345

ABRAMOVITCH R.B., MARTIN G.B., 2004 - *Strategies used by bacterial pathogens to suppress plant defenses.* - Curr. Opin. Plant Biol., 7(4): 356-364.

ANGOT A., ANNETTE V., STÉPHANE G., NEMO P., 2007 - *Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems.* - PLoS Pathog., 3(1): e3.

BARRETT C., CASSELLS A.C., 1994 - *An evaluation of antibi-*

otics for the elimination of Xanthomonas campestris *pv*. pelargonii *(Brown) from* Pelargonium *x* domesticum *cv. 'Grand Slam'explants* in vitro. - Plant Cell Tissue Organ Cult., 36(2): 169-175.

EVRENOSOĞLU Y., MERTOĞLU K., BILGIN N.A., MISIRLI A., ÖZSOY A.N., 2019 - *Inheritance pattern of fire blight resistance in pear.* - Sci. Hortic., 246: 887-892.

FALKINER F.R., 1990 - *The criteria for choosing an antibiotic for control of bacteriain plant tissue culture.* - Int. Soc. Plant Tiss. Cult. Newsletter, 60:13-23.

FALKINER F.R., 1997 - *Antibiotics in plant tissue culture and micropropagation. - What are we aiming at?,* pp. 155-160. - In: CASSELLS A.C. (ed.) *Pathogen and microbial contamination management in micropropagation.* Kluwer Academic Publishers, Springer, Dordrecht, The Netherlands, pp. 371.

KNEIFEL W., LEONHARDT W., 1992 - *Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture.* - Plant Cell Tissue Organ Cult., 29(2): 139-144.

KOHANSKI M.A., DWYER D.J., COLLINS J.J., 2010 - *How antibiotics kill bacteria: from targets to networks.* - Nat. Rev. Microbiol., 8(6): 423-435.

LEIFERT C., CASSELLS A.C., 2001 - *Microbial hazards in plant tissue and cell cultures.* - In Vitro Cell. Dev. Biol., Plant, 37(2): 133-138.

MATHIAS R.J., BOYD L.A., 1986 - *Cefotaxime stimulates callus growth, embryogenesis and regeneration in hexaploid bread wheat (*Triticum aestivum *L em. thell).* - Plant Sci., 46(3): 217-223.

MORGAN D.R., SOLTIS D.E., ROBERTSON K.R., 1994 - *Systematic and evolutionary implications of rbcL sequence variation in Rosaceae.* - Am. J. Bot., 81: 890-903.

MURASHIGE T., SKOOG F., 1962 - *A revised medium for rapid growth and bio assays with tobacco tissue cultures.* - Physiol. Plant., 15(3): 473-497.

NADHA H.K., SALWAN R., KASANA R.C., ANAND M., SOOD A., 2012 - *Identification and elimination of bacterial contamination during* in vitro *propagation of* Guadua angustifolia *Kunth*. - Pharmacogn. Mag., 8(30): 93-97.

PHILLIPS R., ARNOTT S.M., KAPLAN S.E., 1981 - *Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of* Helianthus tuberosus*.* - Plant Sci. Lett., 21(3): 235-240.

QUOIRIN M., LEPOIVRE P., 1977 - *Improved media for i*n vitro *culture of* Prunus *sp.* - Acta Horticulturae, 78: 437-442.

RYU E., 1940 - *A simple method of differentiation between gram-positive and gram-negative organisms without staining.* - Kitasato Arch. Exp. Med., 17: 58-63.

SCHAAD N.W., JONES B.J., CHUN W., 2001 - *Laboratory guide for the identification of plant pathogenic bacteria. Third edition.* - Am. Phytopathol. Soc. Press (APS), St. Paul, MN, USA, pp. 373.

SUN Q., SUN H., BELL R.L., LI H., XIN L., 2011 - *Variation of phenotype, ploidy level and organogenic potential of in vitro regenerated polyploids of* Pyrus communis. - Plant Cell Tissue Organ Cult., 107: 131-140.

SUSLOW T.V., SCHROTH M.N., ISAKA M., 1982 - *Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining*. - Phytopathology, 72(7): 917-918.

VANNESTE J.L., 2000 - *Fire Blight: the disease and its causative agent,* Erwinia amylovora. - CABI Publishing, Wallingford, UK, pp. 370.

ZHU L.H., LI X.Y., AHLMAN A., WELANDER M., 2003 - *The rooting ability of the dwarfing pear rootstock BP10030 (*Pyrus communis*) was significantly increased by introduction of the rolB gene.* - Plant Sci., 165: 829-835.