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Microbial Filtrates Improved Growth Parameters of *In vitro* PVX Infected Potato Plantlets

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study focuses on utilizing the phenomenon of systemic acquired resistance (SAR) to control plant viruses and increased growth in vitro. The study demonstrated the induction of SAR in potato plantlets against Potato Virus X by using bacterial culture filtrates invitro. These biotic inducers included *Pseudomonas spp.* and *Bacillus spp.* The study observed the occurrence of induced resistance and enhanced growth in potato plantlets treated with culture filtrates containing these microorganisms. This was evident through a reduction in PVX infection, disease severity and associated biochemical changes (such as elevated levels of endogenous salicylic acid, protein content, chlorophyll content, peroxidase and polyphenol oxidase activities), as well as improvements in growth parameters.

Keywords: PVX; Bacterial filtrate; micropropagation; growth parameters; protein content.

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1. INTRODUCTION

Microorganisms can contaminate plant tissue cultures, and affect their growth and development. Microbial contamination can occur from various sources, including the environment, contaminated equipment and infected plant material. Once contamination occurs, it can spread rapidly throughout the culture, leading to a loss of genetically uniform plants and production objectives. To prevent microbial contamination, strict aseptic techniques must be followed during all steps of plant tissue culture, including sterilization of tools, media, and plant material. Contaminated cultures should be discarded immediately to prevent further spread of the microbes. In addition to aseptic techniques, the use of antibiotics and antifungal agents in the culture media can help control microbial growth. However, their use should be minimized to avoid potential negative effects on the plants. Regular monitoring of cultures for contamination is crucial to detect early any microbial growth. This can be done through visual inspection, microscopic examination or microbial culture tests. Contaminated cultures should be isolated and treated accordingly to prevent further contamination [1].

Overall, implementing strict aseptic techniques, routine monitoring, and appropriate use of antimicrobial agents are essential for successful plant tissue culture and preventing microbial contamination [2].

Microbial contamination is a major problem in plant cell and tissue culture. Various microorganisms such as fungi, yeasts, bacteria, viruses, viroids, and micro-arthropods like mites and thrips can contaminate plant tissue cultures [1]. In order to combat this contamination, numerous agents have been identified that can induce resistance in plants against pathogens. One effective method involves treating plants with the Bacterial filtrate of non-pathogenic isolates, either individually or in combination. This treatment has been found to be highly effective in inhibiting virus infections through induced systemic resistance [3,4].

The objective of the current study is to identify various microorganisms that can potentially contaminate potato tissue cultures and to determine the most effective bacterial filtrate that can induce growth parameters and systemic resistance in potato cultures against potato virus X in micro propagated potato plantlets.

2. MATERIALS AND METHODS

Plant materials: Potato virus X-infected tuber cv. Spounta cultivated at the Virology Greenhouse Fac. of Agriculture at Ain Shams University served as the starting sprouts for in vitro potato cultures. Polyclonal antibodies were used by DAS-ELISA to test potato tuber for PVX infection [5].

Infected potato sprouts were washed with tap water and immersed in 3% sodium hypochlorite for 20 minutes. Explants were rinsed three times with sterilized distilled water following surface sterilization. Aseptically transferring the explants to MS basal culture media free of hormones [6]. All cultures were raised for three weeks at a temperature of 26°C under 16 hours of light and 8 hours of darkness. As replicates, five sprouts were used. On multiplication MS medium, potato plantlets were *In vitro* sub cultured [7].

Isolation and purification of microbial contaminants: Bacteria that contaminated invitro potato cultures, were inoculated onto nutrient agar medium [8] and cultured at 30°C for three days. For further induced resistance investigations, a single colony was selected on slant tube media and kept at 4°C in a refrigerator.

Characterization and identification of microbiological isolates: Bacterial isolates were identified in accordance with [9] and Bergey's Manual [10].

Induced resistance using bacterial filtrate: According to [11] a loop full of bacterial growth from an agar slope was transferred to 10 ml of tryptic soy broth to prepare the culture filtrate. The bacteria cell was removed from the culture by spinning for 10 minutes at 10,000 rpm. **Bacterial filtrate** (cell-free supernatants) was combined with MS medium in the following ratios: MS medium (as control), 100 ml culture filtrate / 1000 mL MS medium and 200 ml culture filtrate / 1000 mL MS medium. Whereas, MS medium were used (as control). All potato plantlets treated with culture filtrates were incubated under culture conditions in jars with five plantlets per jar and five jars for each treatment.

Evaluation of induced resistance: Induced resistance in potato plantlets against PVX was evaluated based on:

PVX concentration: Percentage of PVX concentration was measured in potato plantlets by DAS-ELISA, to evaluate the degree of induced resistance in potato plantlets against PVX [5].

Plantlets growth parameters: shoot number, shoot length, and growth values of the regenerated cultures plantlets were calculated for each treatment after eight weeks [12].

Protein content: The total soluble protein content of the leaves was determined Folin phenol reagent using according to [13].

Biochemical analysis:

Enzyme activity: Plantlet tissue weighing one gram was mashed in 3 ml of 50 mμ potassium phosphate (pH 7.0). The homogenates were centrifuged for 10 minutes at 12000 rpm at 4^{o}C. The crude enzyme protein (supernatant) was gathered and separated into 1.5 ml. The supernatant was used to estimate the activity of the following enzymes [14].

Peroxidase activity: 200 µl of the crude enzyme dissolved in, 0.1 M phosphate buffer: 3.5 ml (pH 7.0) were mixed with 100 µl of guaicacol (0.251) to measure the peroxidase activity. For five minutes, the optical density at 470 nm was measured once every minute [15].

Polyphenoloxidase activity: was determined in 200 µl of the crude enzyme dissolved in, 0.1 M phosphate **Buffer**: 3.5 ml (pH 7.0) and mixed with 2.95 ml of 20 mM catechol. The optical density was recorded every one min for 5 min at 420 nm [16].

Photopigments: Chlorophyll a, b, and carotenoids were extracted and their concentrations were calculated in accordance with [17, 18].

Salicylic acid: Total salicylic acid was extracted and quantified from potato plantlets by fluorescence HPLC [19].

3. RESULTS

The current study was carried out to induce growth parameters and resistance phenomena via bacterial filtrate through different stages of potato micropropagation. Homologous sprouted tubers were obtained from PVX infected potato plants cv. Spunta.

Infestation of micro propagated potato shoots: Gram negative bacteria (*Pseudomonas spp. and Bacillus spp.*) were among the major genera of bacteria that were tested and were frequently identified in association with micro propagated plantlets. *P. florescence* were identified as more common bacterial contaminant followed by *P. aeruginosa* with the lowest frequency (Table 1).

Induced resistance in micro propagated potato plantlets: Based on the decrease in PVX infectivity and the development rates of potato plantlets, microbial contaminants were screened for biotic inducers. Therefore, the three bacterial contaminants were used to investigate how their culture filtrate affected plantlet growth *In vitro*.

Virus Infectivity: Using specific polyclonal antibodies Potato virus X (PVX) was detected in potato sprouts by DAS-ELISA. Microbial filtrates provided variable reduction in PVX infection. Whereas, individual contaminant filtrate gave high reduction in PVX infection and increasing by increase microbial filtrates concentrations (Table 2).

Table 1. Characteristics and frequency of potato culture-contaminating bacterium isolates

Bacterial contaminants	Characteristics					
	Cell morphology	Motility	Gram stain	Endospors formation	Pigment production	Frequency (%)
P. florescence	Short rod	+	G-	No	greenish yellow	30.88
Bacillus sp.	Long rod	+	G+	Intermediate spore	Creamy	25.46
Pseudomonas aeruginosa	Short rod	+	G-	No	Pale yellow	15.67

G-: Gram stain negative G+: Gram stain positive)

Table 2. Effect of individual microbial filtrate on PVX infectivity in potato plantlets

Parameters / Microbial filtrate	100 MF $^{-1}$L MS medium			200 MF $^{-1}$L MS medium		
	Optical density	% of PVX infectivity	Reduction infection (%)	Optical density	% of PVX infectivity	Reduction infection (%)
P. florescence	0.215	49.5	48.5	0.286	52.45	43.5
Bacillus spp.	0.221	57.0	42.0	0.235	61.45	39.5
P.aeruginosa	0.246	87.0	38.5	0.262	73.40	28.0
Mixed microbial isolates filtrates	0.158	30.25	50.32	0.198	55.42	59.68

Plantlet PVX infected (+ve) = 0.375

Plantlet health (-ve) = 0.125

$$\text{Reduction infectivity (R1)} = \frac{\text{Total plantlets} - \text{treatment}}{Total\ plantlets} \text{ X } 100$$

An average of five jars containing 15-20 plantlets $^{-1}$jar. The microbial filtrate due to reduction of PVX infectivity whereas *P. florescence., Bacillus spp and, P.aeruginosa* revealed the reduction of PVX infectivity (43.5, 39.5 and 28.0) at 100 $^{-1}$L MS medium, respectively and (48.5, 42.0 and 38.5) at 200 $^{-1}$L MS medium, respectively, (Table 2).

Growth rates of potato plantlets: The results of the study indicate that the application of microbial filtrates to potato cultures MS medium resulted in increased growth parameters compared to the control group (healthy and/or PVX-infected potato plantlets). Specifically, the application of bacterial culture filtrates at a concentration of 100 ml/l MS medium led to the highest increase in shoot and root length, as well as the number of (shoots, roots, and leaves). In the case of *pseudomonas florescence* (100 MF^{-1}L) the (shoot length, shoot number, leaves number, root length, root numbers and growth value) was (8.75, 3.22, 5.70, 9.20, 9.75 and 3.25) respectively**.** *P. florescence* gave highest growth parameters compared with other bacterial filtrates (Table 3).

Phytochemical contents: Protein content and enzyme activities were determined in treated potato plantlets with individual and mixed microbial filtrate *in vitro*. Data in Table (3) revealed that, all microbial filtrates inducers increased in protein content and enzyme activities of potato plantlets *in vitro*. The highest protein content was produced by individual microbial filtrate, such as *Bacillus spp.* (1.85); followed by *P. florescence* (175.9), while the lowest content was produced by *P.aeruginosa* (1.41); microbial and mixed microbial Filtrate (1.49 µg/g fresh weight) compared with control (healthy and PVY-infected plantlets, 1.15 and 1.25) respectively.

The highest peroxidase activity was induced by *Bacillus spp* (182.5)., while the lowest was induced by mixed microbial Filtrate (52.3). *Bacillus spp and P. florescence* were also found to induce the highest polyphenoloxidase activity (215.7 and 185.7) respectively. PVX-infected plantlets showed the highest polyphenoloxidase activity (265.3). PVX infection caused a reduction in chlorophyll a, chlorophyll b, and carotenoid contents, but treatment with microbial filtrates increased these contents compared to infected plantlets. Microbial filtrates and PVX infection also increased the salicylic acid content in potato plantlets (Table 4).

Table 3. Effect of microbial culture filtrates on growth parameters of PVX infected potato plantlets

Treatments		Shoot length (cm)	Shoot number	Leaves number	Root length (cm)	Root number	Growth value
Healthy control (disinfectants)		6.35	1.50	5.12	6.75	7.25	1.75
Infected control (disinfectants)		5.86	1.25	3.88	5.25	6.14	1.43
P. florescence	100	8.75	3.22	5.70	9.20	9.75	3.25
MF $^{-1}$L	200	7.95	2.02	5.11	7.98	7.64	2.05
Bacillus spp.	100	7.89	2.96	5.20	7.95	8.89	2.95
MF $^{-1}$L	200	6.25	2.26	4.65	6.56	7.21	1.26
P.aeruginosa	100	6.89	2.70	4.95	7.85	7.05	2.78
MF $^{-1}$L	200	5.69	1.98	3.69	6.02	6.23	1.55
Mixed microbial isolates filtrates 100 MF $^{-1}$L		7.75	2.40	5.15	7.20	8.21	2.50

Calculated as average from 100 potato plantlets (10 jars)

Table 4. Effect of microbial filtrates (100 MF $^{-1}$L) on some phytochemical contents of PVX-infected potato plantlets *In vitro*

Treatments	Protein content (µg/g fresh weight)	Peroxidase specific activity (µg/g fresh weight)	Polyphenoloxidase specific activity (µg/g fresh weight)	Total SA (µg/g fresh weight)	Photopigments (µg/g fresh weight)		
					Chl a	Chl b	Carotenoids
Health control	1.15	64.5	115.2	75.25	2.15	1.49	1.75
PVX-infected control	1.25	168.2	265.3	200.25	1.45	1.21	1.25
P. florescence	1.59	175.9	185.7	150.75	2.27	1.71	1.92
Bacillus spp	1.85	182.5	215.7	239.15	2.75	2.05	2.12
.P.aeruginosa	1.41	154.5	136.2	114.20	2.51	1.91	2.15
Mixed microbial Filtrate	1.49	152.3	170.2	185.50	1.50	1.45	1.75

4. DISCUSSION

As surface sterilizing agents per culture, sodium hypochlorite and ethanol were used to obtain the least amount of contamination in potato sprouts, This findings in agreements with [2].

Using appropriate specialized media, contaminants from potato shoots that had been micro-propagated were separated. Gram-negative bacteria were typically discovered in soil and plant-related environments. Based on the features, the bacterial contaminants were grouped into major genera (*Pseudomonas spp. and Bacillus spp*.). These findings are consistent with those of [1, 20].

It is important to note that the Pseudomonas and Bacillus genera had the highest frequency of bacterial isolates, whereas Enterobacter, Klebsiella, and Corynebacterium genera had the lowest frequency. A number of bacteria were recovered from various plant cultures during tissue culture processes, according to earlier researchers' reports. such as gram positive and gram-negative bacteria were *Staphylococcus xylosus*, *S. aureus*, *S. cohnii*, *Bacillus* sp., *Corynebacterium* sp., *Micrococcus* sp., *Pseudomonas vesicularis*, *Serratia* sp., *Cellulomonas* sp., *Clavibacter* sp., *Curtobacterium*, *Microbacterium* sp., *Acinetobacter* sp., *Wautersia* (*Ralstonia*) and *Stenotrophomonas* sp. [21, 22, 23, 24].

On the other hand, many bacterial contaminants from various plant cultures were isolated during tissue culture methods. These contaminants were identified as *Bacillus spp. and Pseudomonas spp.* [25, 26, 27, 24].

According to research on how bacterial cultures filtrate affected shoots and roots as well as growth value, applications of bacterial filtrate resulted in the longest shoots and roots as well as the highest growth value. It is also important to note that, when compared to the addition of Bacillus culture filtrate, the filtrate of Pseudomonas culture demonstrated a better promotion effect on strawberry growth value. Several studies have shown that *Bacillus and Pseudomonas* created IAA in their cultures in this regard [28,29,30]. Additionally, Acinetobacter, Bacillus, Pseudomonas, Wautersia (Ralstonia), and Stenotrophomonas all produced IAA in [23].

These results revealed that, in tissue culture circumstances, microbial auxins may be encouraging the growth parameters.

In comparison to the control treatment (bacterial filtrate-free), the application of bacterial culture filtrate to the potato culture medium improved all growth metrics for the potato plantlets. It is also important to note that, the filtrate from the (100 MF $^{-1}$L) demonstrated a higher promotion effect on potato plantlet lengths and growth value.

5. CONCLUSIONS

Bacterial Contaminants during tissue culture were isolated using specialized media and grouped into major genera (*Pseudomonas* spp. and *Bacillus* spp.) based on morphological characteristics. Pseudomonas and Bacillus genera have the highest frequency of bacterial isolates, while Enterobacter, Klebsiella, and Corynebacterium have the lowest. Several gram-positive and gram-negative bacteria were recovered from plant cultures during tissue

culture processes. Bacterial cultures filtrate affected shoots and roots as well as growth value. Application of bacterial filtrate resulted in the increase of shoots and roots length as well as the highest growth value. The filtrate of Pseudomonas culture demonstrated a better promotion effect on growth value, compared to Bacillus culture filtrate. Several studies have shown that Bacillus and Pseudomonas created growth promotor in their cultures. The results revealed that microbial auxins may be encouraging all growth parameters *in vitro* and demonstrated a higher promotion effect on potato plantlet growth values.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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