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New surface sterilization protocols for isolation of endophytic bacteria from plants (black turtle beans, peas, and barley)

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New Surface Sterilization Protocols for Isolation of Endophytic Bacteria from Plants (Black Turtle Beans, Peas, and Barley)

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Abstract. The aim of this study was to the isolate the endophytic bacteria, optimize its isolation procedure. Ethanol, sodium hypochlorite, and mercuric chloride at various concentrations and duration were employed to optimize the surface sterilization for the isolation of endophytes from *Phaseolus vulgaris*, *Pisum sativum*, and *Hordeum vulgare*. A total of 21 endophytic bacteria have been isolated from three plants. Combination of 2% sodium hypochlorite, 70% ethanol, and 0.1% mercuric chloride was found effective for the surface sterilization of *Phaseolus vulgaris* and *Pisum sativum*. In case of *Hordeum vulgare*, 70% ethanol and 2% sodium hypochlorite was found suitable for the surface sterilization. Ethanol, sodium hypochlorite, and mercuric chloride were found effective decontaminating agents in optimum condition.

INTRODUCTION

Food is the energy source for everyone around the world, we live in an age where we are growing and producing more food than ever before [1]. All governments are aware of the importance of good quality seed in contributing to increased agricultural productivity and production.

The term endophyte means "inside-plants" and was first coined by De Bary in 1866 [2], Endophytic bacteria have been isolated from many different plant species and almost being found in every plant worldwide [3]. Endophytes are playing an important role to host plants by producing secondary metabolites and some natural products which can improve the plant growth for example phytohormones, such as auxins, cytokinins, and the gibberellins [4], and also induce resistance to herbivorous, drought, parasitism, nitrogen fixation and solubilizing the phosphate [5]. Recently, studies have indicated that endophytic bacteria can increase resistance against pathogens by producing antibiotics. There are a lot of research which study endophytic bacteria and its relation with plants, but until now the interaction between them is not clear and understood [6].

To thoroughly understand the beneficial effects of endophytic bacteria in plants after inoculation, the bacteria must be cultivable under laboratory conditions and this first critical step need a surface sterilization protocol to sterilize the plant parts surface without causing any damage to bacteria in the plant tissues, then isolate the endophytic bacteria from these plants. There are a lot of surface sterilization agents which include sodium hypochlorite, calcium hypochlorite, hydrogen peroxide, ethanol, mercuric chloride, silver nitrate, and others [7]. The sterilization protocol is based on the elimination of contaminants without compromising the biological activity of the plant tissues by using disinfectant solution at suitable concentrations for a specified period.

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MATERIALS AND METHODS

Collection of Plants Samples

The main objectives of our study are comparing different sterilization agents (ethanol, sodium hypochlorite, and mercuric chloride) at various concentrations with a different period in different plants leaves {black turtle beans (*Phaseolus vulgaris*), Peas (*Pisum sativum*), and Barley (*Hordeum vulgare*)}. Then we suggest an efficient sterilization protocol to isolate Endophytic Bacteria from the previous plants.

Plants seeds (black turtle beans, Peas, and Barley) were provided by Ural Federal University, Institute of Natural Sciences and Mathematics, Russia. Plants were germinated and grown on organic soil and at 25 °C with normal conditions.

For the isolation of endophytic bacteria, healthy leaves from plants were collected randomly at the same period for each species (20 days). and used directly for our experimental purpose.

Surface Sterilization

Briefly, the selected leaves were washed and rinsed 3 times by sterile distilled water (SDW) under sterile conditions, then deionized water containing Tween 20® as surfactant (2 drops in 200 mL SDW) used for 45 seconds and then were rinsed several times by SDW in the laminar air flow cabinet. Each isolation procedure was done in triplicate for each plant. We used different sterilizing agents shown in Table 1.

TABLE 1. Different concentration and combination of sterilizing agents with different duration

Sterilizing agent	Duration
70% ethanol (E)	1 min
2% sodium hypochlorite (S)	2 min
0.2% mercuric chloride (M)	30 sec
70% ethanol + 2% sodium hypochlorite (E + S)	Ethanol for 1 min + sodium hypochlorite for 2 min
70% ethanol + 0.2% mercuric chloride (E + M)	Ethanol for 1 min + mercuric chloride for 30 sec
70% ethanol + 2% sodium hypochlorite + 0.2% mercuric chloride (E + S + M)	Ethanol for 1 min + sodium hypochlorite for 2 min + mercuric chloride for 30 sec

Confirm the Disinfection Protocol

Validation of the surface sterilization procedure was done by two methods, first by culturing aliquots of the sterile water from the last rinsing onto nutrient media agar, second by imprinting the surface sterilized plant tissue on nutrient media agar also [8]. Then the petri dishes Incubated at 28 ºC for 15 days to check the growth of bacterial colony.

Media for Isolating Endophytic Bacteria

Nutrient agar media with nystatin (antifungal) at a concentration of 30 µg/mL to suppress the growth of fungi was used [9]. The leaves were ground with 6 mL of aqueous solution (0.9% NaCl) using a sterile mortar and pestle.

After 18–24 hrs of incubation period, we selected morphologically different bacterial colonies. The process of isolating the bacteria was repeated several times to get pure colonies then they were stored at 4 °C till further used.

Statistical Analysis

Each step was done in triplicates and SD represents standard deviation. All the samples were prepared using double deionized Millipore water.

RESULTS

To check the efficiency of sterilizing agents and survival percentage of treated leaves plants, we compared between the chemical agents individually, and the different combination for a specific time. Our results showed that the explants were found contaminated with a high percentage, when we used the sterilization agents individually. For the surface sterilization and the percentage survival of leaves after treatment of *P. vulgaris* by the sterilization agents, the combination of 70% ethanol, 2% sodium hypochlorite and 0.2% mercuric chloride was found effective (Figure 1). But using those combination for *P. sativum*, the plant died, and they damaged the leaves. Therefore, 70 % ethanol and 0.2% mercuric chloride were more suitable because they increased the survival percentage of plant (Figure 1). Also the results for the surface sterilization of *H. vulgare* showed that 70% ethanol and 2% sodium hypochlorite were the best combination.

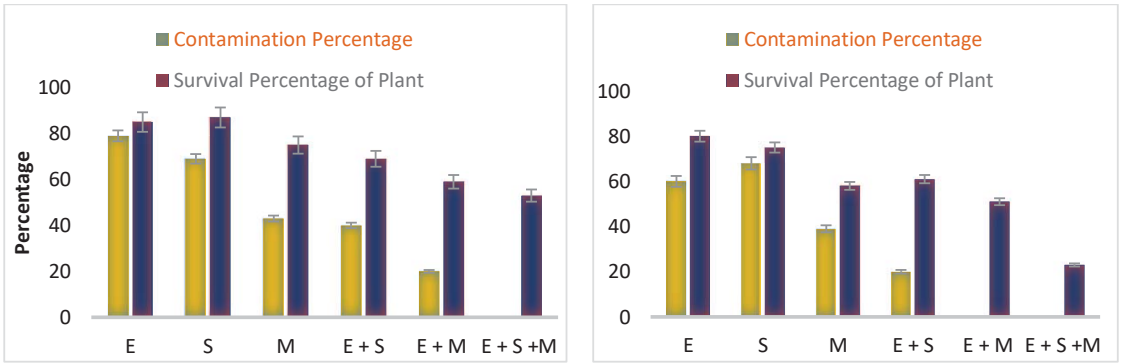

FIGURE 1. Optimum condition for the surface sterilization of *Phaseolus vulgaris* (a) and *Pisum sativum* (b). Error bars show standard errors

To consider that surface sterilization is complete, and isolated bacteria are endophytic, they must be no bacterial colony in the control samples. So, we checked the sterility, by culturing aliquots of water from the last rinsing onto nutrient media and imprinting the surface sterilized plant tissue onto nutrient media. The results under optimal condition showed no microbial growth on the media for all samples.

Nutrient agar was used for the isolation of endophytic bacteria. About twenty-one pure endophytic bacterial colonies were isolated from our three plants. We selected thirteen isolates from the total isolates for further investigation.

DISCUSSION AND CONCLUSION

As its known, endophytic bacteria exist in small numbers in plants if its compared with bacterial pathogen or rhizospheric bacteria, and the surface of plants (e.g., roots, stems, leaves, seeds, and fruits) carries a diverse range of bacteria. For the isolation of endophytes which colonize the internal tissue of plants without any contamination with the other bacteria that are located on the surface, the effectiveness of surface sterilization process must be completely success prior to inoclating the explants onto the nutrient medium.

In our study, we used different chemical disinfectant (ethanol, sodium hypochlorite, and mercuric chloride) for surface sterilization of leaves from *Phaseolus vulgaris, Pisum sativum, and Hordeum vulgare*. The results showed, using the treatments by the sterilizing agents individualy did not give the efficiency to reduce the contamination percentage, but in other case using different combination of sterilizing agents succes to reduce the contamination.

This work may be the first report on endophytic bacteria from *P. vulgaris*, *P. sativum*, and *H. vulgare* form Sverdlovsk region, Russia.

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REFERENCES

1. K. Havas and M. Salman, International Journal of Food Safety Nutrition and Public Health **4(1),** 4–11 (2011).
2. A. De Bary, *Handbook of Physiological Botany* (Springer-Verlag, Leipzig, Germany, 1866).
3. C. Mastretta, S. Taghavi, D. Van der Lelie, A. Mengoni, F. Galardi, and et al., Int. J. Phytorem. **11**, 251–267 (2009).
4. N. Eevers, M. Gielen, A. Sánchez-López, S. Jaspers, J. C. White, and et al., Microb. Biotechnol. **8**, 707–715 (2015).
5. A. V. Sturz, B. R. Christie, B. G. Matheson, and J. Nowak, Biol. Fertil. Soil **25**, 13–9 (1997).
6. K. Ulrich, A. Ulrich, and D. Ewald, FEMS Microbiol. Ecol. **63**, 169–180 (2008).
7. S. Barampuram, G. Allen, and S. Krasnyanski, Plant Cell Tissue and Organ Culture **118** (2014).
8. A. J. McInroy, and J. W. Kloepper, *Improving Plant Productivity with Rhizosphere Bacteria* (CSIRO, Melbourne, Australia, 1994).
9. MERCK (2000) Microbiology Manual.1.