Effect Of Osmotic Stabilizer On Dark-Septate Endophytic Fungus *Veronaeopsis Simplex* Protoplast Regeneration

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Abstract

The hyphomycete, *Veronaeopsis simplex*, belongs to the group of so-called dark septate endophytes (DSE) colonize root plant intra and intercellulary without causing harm for the plant host. Knowledge about fungus interaction with the host plant is little. This paper describes the influence of different cultural conditions on protoplast isolation and regeneration from *V. simplex* fungus. Current need for genetic engineer and the absence of reports have initiated this research to target development of an efficient and reproducible protocol to make this fungus amenable for further studies. The mycelia age, osmotic stabilizer and lytic enzyme were the main factor that affected the protoplast formation. The maximum yield of protoplast was 3.2 x 106/g fresh mycelia was obtained from 1 week old mycelia digested with the mixed of 1.5% cellulose, 1 % macerozyme and 1.5 % lysozyme for 3 h incubation at 37^{o}C in the dark. Morphology and structure of protoplast released were observed using microscope. The result showed that CMMY and PDA medium supplemented with 0.6 M sucrose as osmotic stabilizer have supported highestt regeneration ability of the protoplast compared to KCl or mannitol. Thun, it is believed that an important foundation has set for efficient genetic manipulation of this fungus.

Keywords: DSE, isolation, regeneration, protoplast, *Veronaeopsis simplex*

1. Introduction

Veronaepsis simplex is a species that belong to dark-septate endophytes (DSE) fungi characterized by melanized intercellular and intracellular runner hyphae and so-called microsclerotia (aggregation of dark, thick-walled, closely packed inflated cells) within epidermis and cortex of plant roots. Much attention is now being paid for DSE fungi for the unique characteristics, chemistry, bioactivity and the relationship between fungal endophytes and their host plants (Tan & Zou 2001l;Tao *et al* 2008) in particularly *V. simplex.* Based on the previous research, the fungi showed potential ability on plant growth promotion and disease suppression (Khastini *et al* 2002). Concerning to the facts above, it is important to elucidate the mechanism involved. Establishing an effective protocol to obtain and regeneration protoplast can be considered as alternative way to study the functional analyses mechanisms related to fungus-plant interaction.

In general, protoplasts are referred to as the first organized body of a species that had its cell wall completely or partially removed using either by mechanical or enzymatic means (Peberdy and Ferenczy, 1985; Homolka, 1988). Protoplast contain all the intracellular organelles of a cell and form a vital link in transfer of micro-molecules between cyto-organelles (Peberdy & Ferenczy, 1985). The isolated protoplasts may be excellent materials for plasmid DNA transformation or mutagenic treatments and in a variety of genetic manipulation techniques targeting specific genetic information transfer into the fungal speies for the development of modified strains. In fungi, protoplasts can be defined as spherical cells whose chitinous cell walls have been removed by appropriate digestive enzymes. The successful and optimizing the production of protoplast can be achieved by knowing which determinant factors such as age and quantity of mycelium, osmotic stabilizer, temperature, pH, digestion time, and lytic enzyme concentration (Peberdy 1976; Chadegani *et al* 1989). Protoplast which consists of cell wall reconstitution then will regenerate and return to mycelia growth.

Some protocols to obtain and regenerate the protoplasts have already been established for some fungi and Basidiomycetes species, such as *Agaricus bisporus* (Royer *et al* 1992) and *Suillus granulates* (Dias *et al* 1996). Little is known about the protocol to obtain protoplast from DSE fungi. Even though the methods for the release and regeneration of protoplasts are available, there is no single methods which generally applicable for all fungi. There are many factors such as lytic enzymes, osmotic stabilizers, mycelium age and the type of microorganism which can affect the protoplasts production (Peberdy 1995). In this paper the influenced factors will be limited and focused on the effect of three osmotic stabilizers on protoplast production. Osmotic stabilizers are

important to protect protoplast from being broken during enzymatic actions and there are no recommendations about kinds of suitable osmotic pressure stablizer and their appropriate concentration to be used in certain fungus spesies.

2. Materials and Methods

2.1 Fungal isolate

*Veronaeopsis simplex (*MAFF240802) isolated from a soil sample collected from wooded areas Japan (available from Dr. K. Narisawa). To prepare the inoculums, the isolate was grown on CMMY medium (cornmeal, infusion form [Difco, Detroit, Michigan], 25 g; malt extract [Difco], 10 g; yeast extract [Difco], 2 g; Bacto agar [Difco], 15 g, 1 L H2O) in Petri dishes (90 mm diam) at room temperature (about 23 ^{o}C).

2.3 Protoplast production

Production of fungal protoplast in this experiment was attempt using Hasiba method (1992). Mycelial discs of 3 mm size from *V. simplex* grown on CMMY medium were inoculated into 50 ml CMMY broth in 250 ml conical flask and incubated at 30 oC. The age of mycelium plays an important role in the successful of protoplast production. A set of experiment was carried out to determine the optimum age of mycelium for the release of protoplast. The mycelia were separated from the broth culture at different time intervals (1, 2 and 3 week) and washed in 3-4 changes of sterile distilled water for removing the broth. The mycelia were then incubated with constant stirring at 100 rpm with the 1.5% cellulose, 1 % macerozyme and 1.5 % lysozyme for 3 h incubation. The mycelia were separated from the incubation mixture by centrifugation and filtration. Three kinds of osmotic stabilizers were selected in this experiment: potassium chloride, mannitol and sucrose, which were all tested at concentrations of 0.6, 0.8 and 1.0 M in order to determine the most suitable osmoticum. The appropriate osmotic stabilizers and concentrations were determined. According to the results of the other factors (data not shown), the protoplast liberation was established that under osmotic stabilizers which dissolved the enzyme was 0.6 M sucrose, and the temperature was 37°C. The obtained protoplasts resuspended in 1 ml osmotic buffer and the number of protoplast counted on a haemocytometer and examined under a light microscope (Olympus BX51).

2.4 Protoplast regeneration

Protoplasts were regenerated in soft CMMY and PDA medium agar. Osmotically stabilizer that were used for regeneration of protoplast: KCl, mannitol and sucroce. Protoplast were maintained in the plates and incubated for 48 h in the dark. The regeneration abitily of protoplast were counted.

3. Result and Discussion

3.1 Protoplast production

Figure 1 show the protoplast of *V. simplex* that being release and size were varied. This is may resulted from the release of protoplast from different region of the hyphae, Maximum yield of releasing protoplast was 3.2 x 106/ g wet mycelia obtained from 1 week old mycelia while 2 and 3 weeks old mycelia released 2.4 x 10 4/g and 5.0 x 10 4/g respectively Both juvenile and older mycelia of *V. simplex* could be used for protoplast isolation and regeneration. But the effective growth stage for the formation of protoplast is in the early of mycelia growth phase. Generally dark

septate endophytic fungi unlike other fungi have rather slow growth rates and for *V. simplex* showed the same ones.

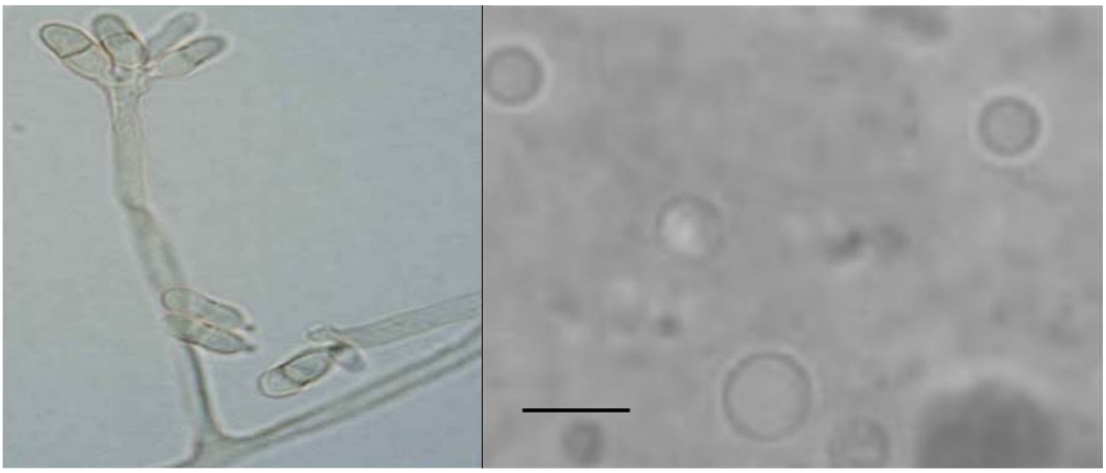

Figure 1. The morphology and protoplast of *Veronaeopsis simplex* that being release. Bar 10 µm

Increasing in age of mycelia (2 and 3 weeks old) will affected on the less number of protoplast released, perhaps reflecting the senescence of large part of mycelia mats (Rui and Morrel

1993). The presence of rigid cell walls which is important structure to fungal hyphae makes difficulty in protoplast liberation. In the early growth phase, the mycelia are sensitive to the enzyme which will make lytic enzyme more effective for cell wall degradation. Protoplast will be released after 3 hour incubation. This is consistent with the previous studies which have employed younger cultures to avoid this problem and enhance protoplast yield (Trojanwski & Hutterman 1984; Eguchi *et al* 1990)

Lytic enzyme is also important factor that affecting protoplast release. Lytic enzymes that used in this experiment have successfully been used to liberate the protoplast. The concentration used was also effective. If the enzyme concentration used was too low, mycelia can not be digested sufficiently. If too high concentration used, protoplast membrane will be injured in spite of high yields, which will lead to a low regeneration frequencies (Kitamoto *et al* 1984). Osmotic stabilizer is another factor that influencing protoplast liberation. An osmotic stabilizer is only suitable for few species respect to the difference of cell wall composition in different species (Chen & Belanger 2000). Sucrose 0.6 M was selected from three available osmotic stabilizer and analyzed for protoplast liberation.

3.2 Protoplast regeneration

As protoplasts have lost their cell wall because of enzymatic actions, use of osmotic stabilizers is important to protect protoplasts from being broken and further benefit in improving enzyme activities. Of course, up to now, for a certain fungus species, there are no reasonable explanations about which kind of chemical composition are more suitable osmotic pressure stabilizers than others regarding favouring protoplast regeneration (Wubie *et al* 2014*)*.

Protoplast regeneration provides a relative measure of the effects of enzyme treatment on cell viability. Protoplasts that lack the ability to regenerate presumably either lack nuclei or were damaged at some point during or after the enzyme treatment. In contrast with protoplast release, regeneration improved with increasing age of the cultures. The osmotic stabilizer supplemented in the media also appeared to influence protoplast regeneration. Sucrose that added on both PDA and CMMY media was proved as the best osmotic stabilizer with 1.3% and 1.5% regeneration ability respectively (Figure 2). Sucrose also used as osmotic stabilizer in protoplast regeneration of *A. bisporus and A. bitorquis* (Yanagi *et al* 1985; Sonneberg *et al* 1988) . There is a speculation regarding to the osmotic stabilizer used, which act as a direct precursor of cell wall synthesis or the

indirect one by metabolism and thus could speed up the cell wall synthesis influence the protoplast regeneration (Li *et al* 2010)

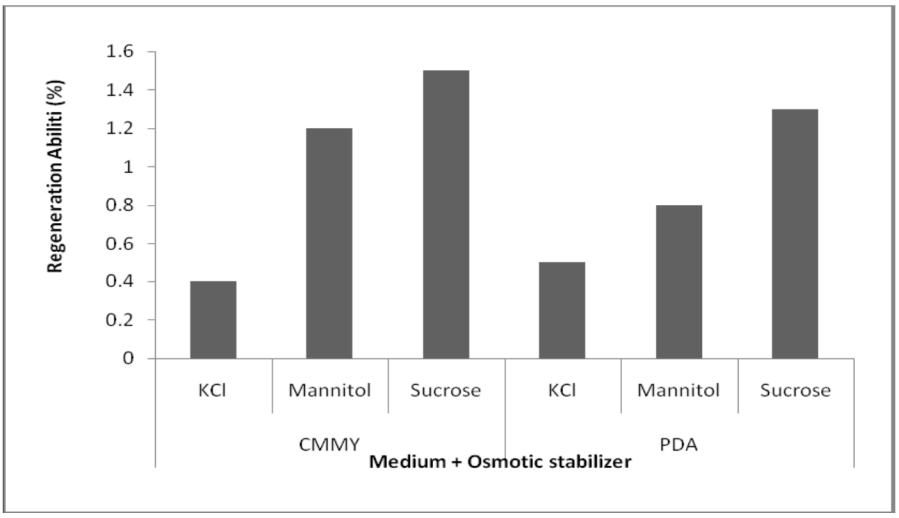

Figure 2. Regeneration ability of protoplast on different medium and osmotic stabilizer added

4. Conclusion

There are very vew reports earlier on the isolation and regeneration of protoplast for dark septate endophytic fungus. These preliminary studies results indicated that viable protoplast from DSE fungus *V. simplex* successfully isolated and can be regenerated. This study has laid the foundation for further study in order to reveal the unknown mechanism of the V. simplex role in the plant host.

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