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Influence of Physiological and Environmental Factors on Growth and Sporulation of an Antagonistic Strain of *Trichoderma viride* RSR 7

R. K. Jayaswal, Rajesh Singh[1] and Youn Su Lee[2]*

Department of Biological Sciences, Illinois State University, Illinois, IL, U.S.A.
[1]Centre of Advanced Studies in Botany, Banaras Hindu University, Varanasi 221 005, India
[2]Division of Applied Plant Sciences, Kangwon National University, Chuncheon 200-092, Korea
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Influence of physiological and environmental factors on an antagonistic strain of *Trichoderma viride* RSR7 were studied optimize its biocontrol potential. The growth and sporulation of *T. viride* was greatly influenced by various carbon and nitrogen sources, and the environmental factors such as pH and temperature. The best growth and sporulation of *T. viride* was observed when sucrose, peptone and trehalose were supplemented in the medium as sole carbon sources. Rhamnose, pyruvic acid and sorbitol also supported a good growth. However, with these carbon sources the sporulation was poor. Growth and sporulation was also affected by various nitrogen sources. Growth and sporulation both were favoured by ammonium forms of nitrogen compared to nitrite or nitrate forms. Urea did not support either growth or sporulation. Among amino acids, glutamic acid, asparagine, leucine, aspartic acid, glutamic acid and alanine supported good growth as well as sporulation. *T. viride* was able to utilize large number of amino acids as sole nitrogen source. Proline was good for growth, but not for sporulation. Maximum growth and sporulation of *T. viride* was between pH 4.5 to 5.5. Temperatures between 20°C and 37°C were good for both growth and sporulation of *T. viride*. At lower temperatures (i.e. below 20°C) growth and sporulation were inhibited. Based on the present study it may be concluded that *T. viride* RSR7 is capable of growing and sporulating with varied nutritional and environmental conditions and, therefore, this strain of *T. viride* may be useful as a biocontrol agent under diverse physiological and environmental conditions.

KEYWORDS: Antagonism, Physiological and environmental factors, Sporulation, *Trichoderma viride*

The species of *Trichoderma* are distributed worldwide and share a common habitat with a variety of pathogenic and non-pathogenic microorganisms. *Trichoderma* species have been of great interest because of their ability to produce cellulases (Mandels, 1975) as well as their roles in biological control of plant pathogens (Cook and Baker, 1983; Papavizas, 1985) and nematodes (Cook and Baker, 1983; Yang *et al.*, 1976), and plant growth promotion (Chang *et al.*, 1986). There have been several successful attempts to introduce *Trichoderma* spp. in soil for biological control of plant pathogens (Papavizas, 1985; Chet, 1990; Hjeljord and Tronsmo, 1998) and nematodes (Siddiqui and Mahmood, 1996). However, their utility as a biocontrol agent is sometimes limited by inhospitable conditions of soil (Papavizas, 1985) due to physiological and environmental conditions. Detailed studies on such inhospitalities are lacking.

Because of the known role of *Trichoderma* as a biocontrol agent and also as cellulase producer, there have been a few attempts *in vitro* on the physiological and the environmental factors which limit the growth of this antagonistic organism (Betina and Farkas, 1998; Kubicek-Pranz, 1998). Knowledge on these factors may lead to a better understanding of the population dynamics of *Trichoderma* in soil and other habitats. Also, studies on the factors affecting growth and sporulation of *Trichoderma in vitro* can be useful if the information derived from such studies can be used to the technology of large scale production of biomass for use in biocontrol. Although, there is extensive literature on the enzymes and biological control potential of *Trichoderma*, there are a few studies on their ecological requirements in relation to their growth and sporulation. Krystofova *et al.* (1998) have studied the effect of phophoinositides and inositol phophate on growth and photoconidiation in *Trichoderma viride*. Danielson and Davey (1973a, b), Shukla and Mishra (1970), and Tye and Willet (1977) have studied nutritional aspects of *Trichoderma*, and Danielson and Davey (1973a, b), and Macauley and Griffin (1969) have studied their CO_2 and pH responses. It has been also reported that the sporulation varies with light and darkness (Miller and Reid, 1961). In spite of enormous research on *Trichoderma* spp. as a biocontrol agent (Chet, 1990; Cook and Baker, 1983; Papavizas, 1985) their physiological and environmental requirements have not been studied in depth.

We isolated an antagonistic strain of *Trichoderma viride* RSR7, which was capable of antagonizing a large number of fungi. We have studied the nutritional requirement of this strain by selecting a large number of carbon and nitrogen sources. We have also studied the growth

*Corresponding author <E-mail: younslee@kangwon.ac.kr>

and sporulation of this strain under different pH and temperature regimes.

Materials and Methods

The culture of the *Trichoderma viride* RSR7 was maintained on potato dextrose agar (PDA) or corn meal agar (CMA) slants at 4°C. For physiological studies M9 medium (Miller, 1972) with following composition was used: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; distilled water 1000 m*l*. M9 salts and agar were autoclaved separately and then supplemented with 2 m*l* of 1 M $MgSO_4 \cdot 7H_2O$, 10 m*l* of 20% glucose, 0.1 m*l* of 1 M $CaCl_2$ and 0.5 m*l* of vitamin B_1 (thiamine hydrochloride). The pH of the medium was adjusted to 6.5 unless otherwise mentioned.

Effect of carbon sources. The carbon sources used in this study were arabinose, citric acid, fructose, galactose, glucose, inositol, lactose, malic acid, maltose, mannitol, mannose, poly galacturonic acid (PGA), pectin, peptone, rhamnose, ribose, sodium acetate, sodium citrate, sodium succinate, sorbitol, starch, succinic acid, sucrose, trehalose and xylose. These carbon sources were added to M9 medium at a final concentration of 0.2% (w/v) in Erlenmeyer flasks separately before autoclaving. The media containing the carbon sources were poured in triplicate set of Petri dishes and were allowed to solidify. The Petri dishes containing solidified medium were centrally inoculated with 5 mm blocks from actively growing cultures of *T. viride* RSR7. The plates were incubated at 25±2°C. The radial growth of *T. viride* was measured at an interval of 72 and 120 hrs. Sporulation was also recorded at the same time on the basis of the colour intensity developed in the colonies as a result of sporulation. It was graded as poor (+), good (+) and excellent (+++).

Effect of nitrogen sources. The nitrogen sources were calcium nitrate, potassium nitrite, potassium nitrate, sodium nitrite, sodium nitrate, ammonium sulphate, ammonium biphosphate, ammonium chloride, ammonium nitrate, and urea. These nitrogen sources were amended at the rate of 0.1% (w/v) in sets of conical flasks containing 100 m*l* of M9 medium. These sets were autoclaved at 15 lb pressure for 20 min. Thereafter, the cooled and melted medium was poured separately in sterilized Petri dishes. After solidification 5 mm blocks of actively growing cultures of *T. viride* RSR7 were centrally inoculated on the medium. The plates were incubated as described earlier and the radial growth of *T. viride* was recorded at an interval of 72 and 120 hrs. The sporulation was recorded as described above.

Effect of amino acids. Different amino acids selected for the study were alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, praline, serine, threonine, tryptophane, tyrosine, and valine. One hundred ml M9 medium was prepared in sets of 250 m*l* Erlenmeyer flasks. Each amino acid was amended (0.1% w/v) in sterilized and cooled (at 40°C) M9 medium separately. The inoculation of *T. viride* RSR7 for radial growth and sporulation was done as described above. The radial growth and sporulation were recorded at an interval of 72 and 120 h.

Effect of pH. Different pH levels selected for the study were 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. One hundred m*l* M9 medium was prepared in sets of 250 m*l* Erlenmeyer flasks. The pH of the medium was adjusted with NaOH or HCl in triplicate sets before autoclaving. The procedure for inoculation and measurement of the radial growth of the test fungus was similar as described above.

Effect of temperature. Sterilized and cooled M9 medium was poured in triplicate sets of Petri dishes. The plates with the solidified medium were inoculated with 5 mm agar block from the actively growing colonies of the test fungus. The inoculated Petri dishes were incubated at 18, 24, 30, 37, and 42°C temperatures separately for 5 days. The radial growth of *T. viride* was measured at an interval of 72 and 120 hrs. The sporulation was recorded at the same time as described above.

Results

The growth and sporulation of *T. viride* was greatly influenced by various carbon and nitrogen sources (Figs. 1, 2, 3). The best-liked sources were peptone, trehalose and sucrose with which both growth and sporulation were excellent. The radial growth was sparse and very fast with citric acid and succinic acid. However, with these carbon sources *T. viride* did not sporulate well. The carbon sources, which supported both growth and sporulation very well, were maltose, glucose, mannose, fructose, ionositol, sodium acetate, galactose and starch. Growth and sporulation of *T. viride* was good when sodium citrate, xylose, arabinose, lactose, pectin, PGA, ribose and malic acid were supplemented in the medium. In a few cases the growth was supported well but the sporulation was poor. These carbon sources were rhamnose and sorbitol.

Various nitrogen sources also affected the growth and sporulation of *T. viride*. The form of nitrogen was important factor. Both sporulation and growth were favoured by ammonium forms of nitrogen than nitrite or nitrate forms. Also, with ammonium forms of nitrogen the sporulation was heavy. With calcium nitrate *T. viride* neither grew well nor sporulated well. There was a poor growth when

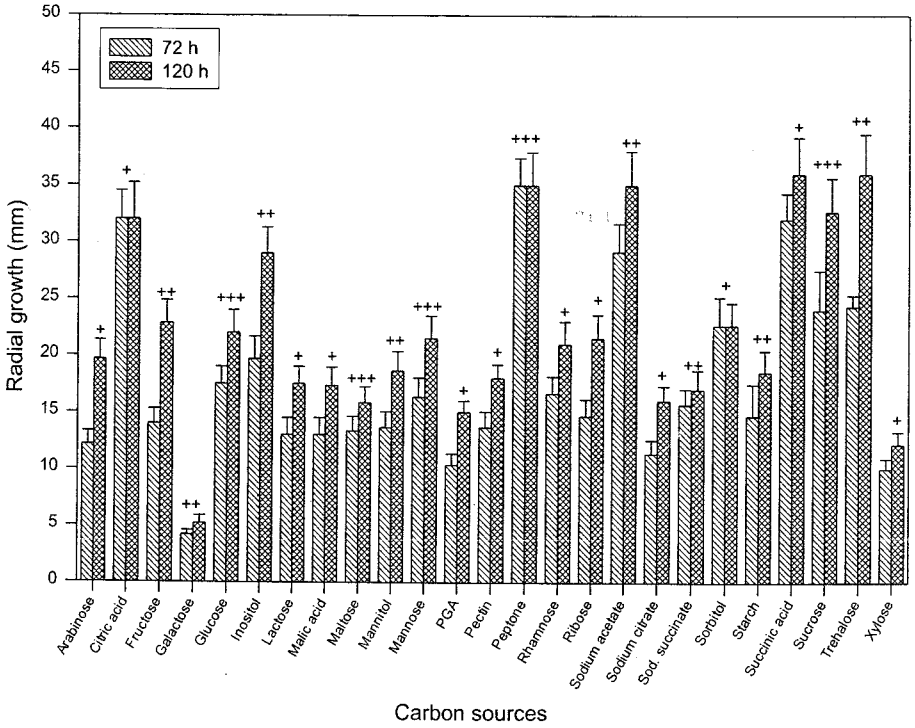

Fig. 1. Effect of carbon sources on the radial growth and sporulation of *Trichoderma viride* RSR7 at 72 and 120 hours of incubation period at 25±2°C. +, poor sporulation; ++, good sporulation; +++, excellent sporulation. Error bars represent standard error.

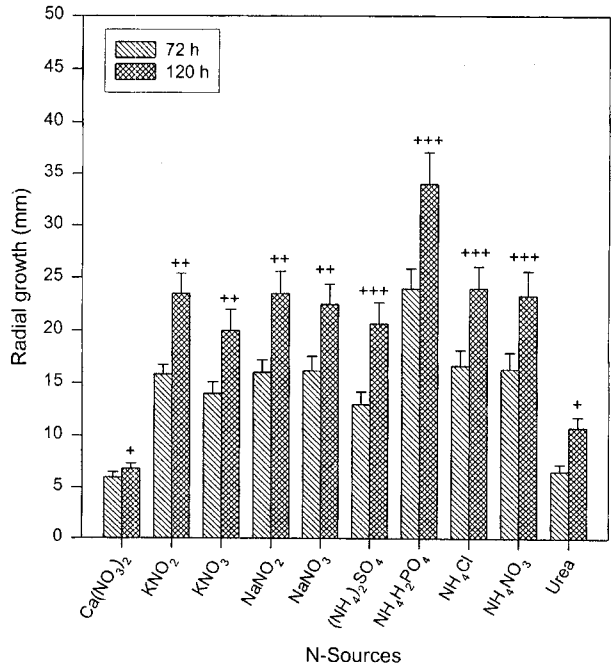

Fig. 2. Effect of various inorganic nitrogen sources and urea on the radial growth and sporulation of *Trichoderma viride* RSR7 at 72 and 120 hours of incubation period at 25±2°C. +, poor sporulation; ++, good sporulation; +++, excellent sporulation. Error bars represent standard error.

this was used as a sole nitrogen source. Urea also did not support the growth and sporulation of *T. viride*.

With single amino acid as a sole source of nitrogen, there was again a great variation in the growth and sporulation. On the basis of their effects the amino acids may be grouped under the following categories in decreasing order: i) glutamic acid, asparagine, leucine, aspartic acid, glutamine and alanine (these amino acids supported good growth as well as sporulation); ii) glycine, valine, serine, tyrosine, and phenylalanine (supported good growth as in 1, but the sporulation was lesser than them); iii) histidine, cysteine, tryptophane, lysine, threonine, arginine, methionine (growth and sporulation were moderate); and iv) proline (growth was good but sporulation was very poor).

Optimum growth and sporulation of *T. viride* was recorded between pH 4.5 to 5.5 (Fig. 4). The growth and sporulation decreased with either decreasing or increasing the pH below 4.5 or above 5.5, respectively. At pH 3.5 and 4.0 the growth of *T. viride* was still good but the sporulation was reduced. Above pH 6.0 with increasing the pH the growth and sporulation decreased proportionately. At pH 8.0 and 8.5 the growth and sporulation were very poor. At pH 9.0 no growth of *T. viride* could be observed. *T. viride* showed a high range of temperature tolerance. It grew and sporulated well between temperatures 20 to 37°C (Fig. 5).

Discussion

Trichoderma viride showed an ability to use a variety of carbon as well as organic and inorganic nitrogen com-

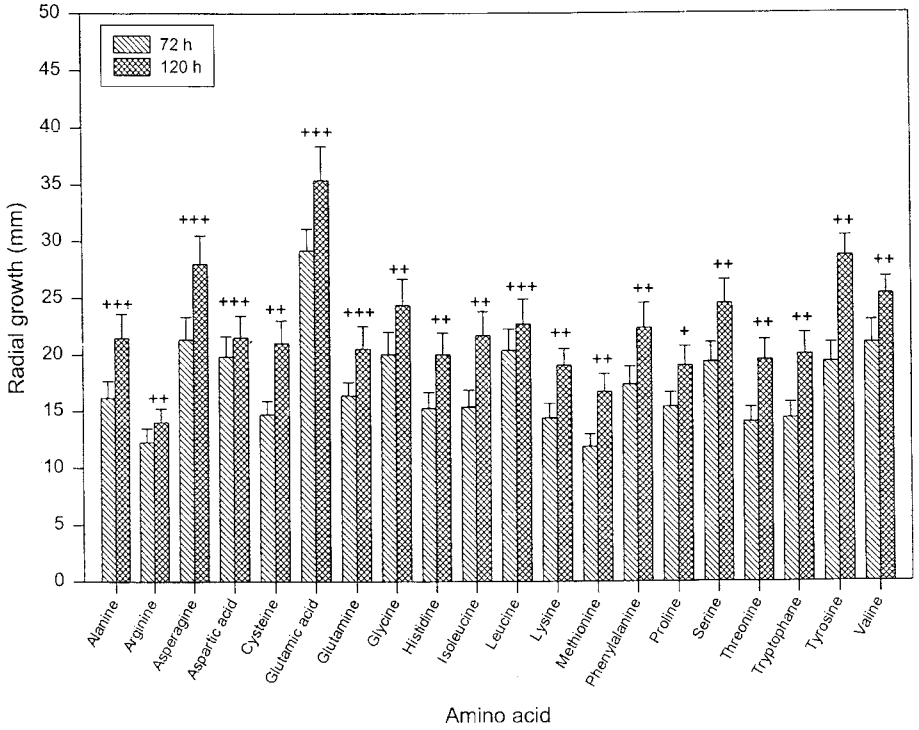

Fig. 3. Effect of amino acids on the radial growth and sporulation of *Trichoderma viride* RSR7 at 72 and 120 hours of incubation period at 25±2°C. +, poor sporulation; ++, good sporulation; +++, excellent sporulation. Error bars represent standard error.

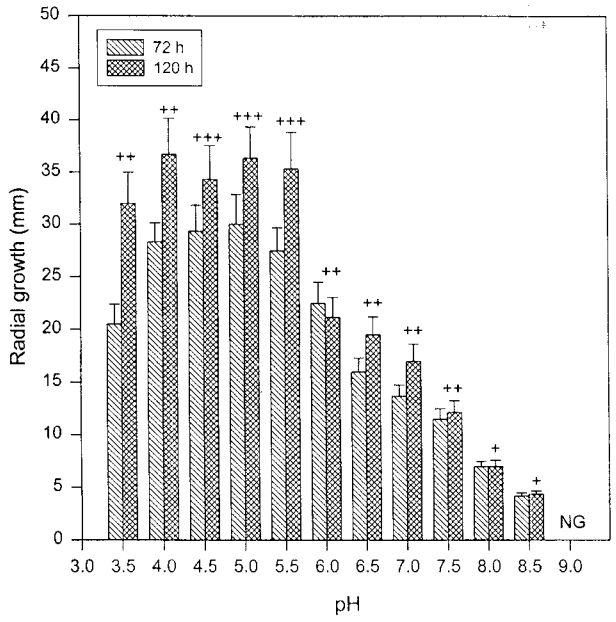

Fig. 4. Effect of pH on the radial growth and sporulation of *Trichoderma viride* RSR7 at 72 and 120 hours of incubation period at 25±2°C. +, poor sporulation; ++, good sporulation; +++, excellent sporulation. Error bars represent standard error.

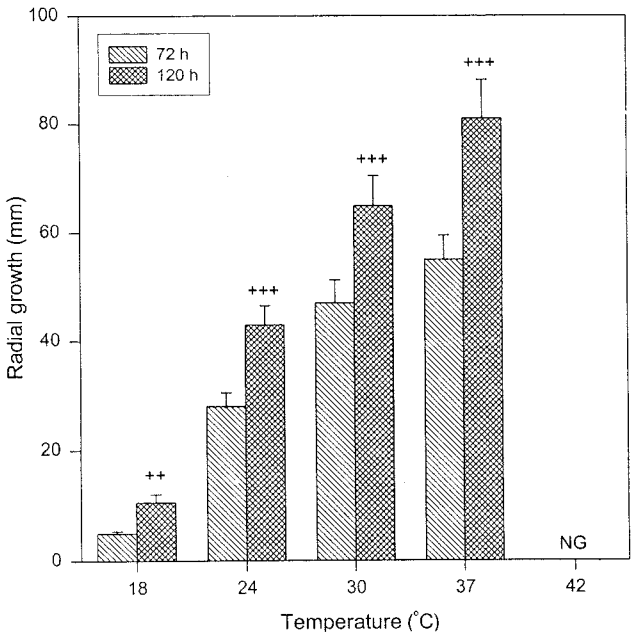

Fig. 5. Effect of various temperatures on the radial growth and sporulation of *Trichoderma viride* RSR7 at 72 and 120 hours of incubation period. +, poor sporulation; ++, good sporulation; +++, excellent sporulation. Error bars represent standard error.

pounds as sole source of carbon or nitrogen. Papavizas (1985) has stated that different species of *Trichoderma* have their own ecological preferences. However, the species of *Trichoderma* are distributed worldwide. From our study it is evident that the wide occurrence of *T. viride* is supported by the fact that it has an ability to utilize a variety of nutritional factors as well as they have a broad range of pH and temperature tolerance for their growth

and sporulation. Although with certain conditions the growth and sporulation were reduced, but *T. viride* was still able to grow. The only condition we found at which *T. viride* was unable to grow was pH 9.0 and above. These observations are interesting for the ecological behavior of this antagonistic fungus. We have earlier reported that *Trichoderma* spp. prefer and grow well in the soils having acidic pH and high organic matter (Upadhyay and Rai, 1978, 1979).

Maximum growth of *T. viride* was recorded when peptone was used in the medium. This superior growth in peptone among nitrogen compounds may be attributed to its being a complex mixture of peptides and amino acids containing some water-soluble vitamins (Cochrane, 1958). Other workers have also reported that fungi are favoured by peptone as a source of carbon in the medium. Among amino acids aspartic acid and asparagine were good for the growth and sporulation of *T. viride*. These amino acids have been reported as good sources for fungal growth (Chattopadhyay and Nandi, 1981).

It was interesting to note that *T. viride* preferred ammonium forms of nitrogen. This may be important for *T. viride* as biocontrol application in relation to agricultural practices. Ammonium fertilizers are commonly used in agronomic practices. Therefore, information on these factors affecting the population dynamics of this biocontrol agent will be useful if the system is to be used for biocontrol of fungal pathogens where *T. viride* is a component. We are unable to explain why the growth and sporulation was favoured by ammonium forms of nitrogen. However, it has been observed by Nicholas (1965) that ammonium ions can diffuse more quickly into the cells and then is utilized quickly. Other explanation for better growth of *T. viride* with ammonium forms of nitrogen may be because of the fact that uptake of ammonium nitrogen reduces the pH of the surrounding (MacNish, 1988). In addition, *T. viride* grows better when the pH of the medium is lowered (Fig. 4), this situation provides *T. viride* a better opportunity to grow. This lowering of pH due to quick and efficient ammonium uptake (Nicholas, 1965) may have contributed to a better growth and sporulation of *T. viride* with ammonium forms of nitrogen. Simon and Sivasithamparam (1988b) have proposed that application of ammonium sulphate results into an increased activity of *Trichoderma* spp. with a consequent increase in suppression of *Gaeumannomyces graminis* var. *tritici*. When ammonium treated soil was limed to increase the pH a reduction in the activity of *Trichoderma* occurred. Therefore, it seems obvious that efficient utilization of ammonium forms of nitrogen is related to pH changes in the medium, which may be a reason for an increased growth and sporulation of *T. viride*. It has also been observed that elevation of soil pH results into increased severity of several diseases (Cook and Baker, 1983; Simon and Sivasithamparam, 1988b, 1990). This may be correlated to a decreased activity of antagonists like *Trichoderma*, which form some times up to 70% of the total number of fungi in certain soils (Simon and Sivasithamparam, 1988a, c).

A broad range of temperature tolerance for growth and sporulation of *T. viride* (i.e. 20 to 37°C) is another interesting feature for the suitability of this antagonist as a biocontrol agent. The biocontrol formulations developed from this isolate of *T. viride* may be used in a wide range of geographical locations because of its versatility to utilize a large number of carbon and nitrogen sources and its broad spectrum of pH and temperature tolerance.

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