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GROWTH REQUIREMENTS OF RHIZOCTONIA REPENS M 32

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Abstract

Rhizoctonia repens M 32, a mycorrhizal isolate from *Orchis militaris* requires both a carbohydrate (glucose or sucrose) and an amino acid (aspartic acid, glycine, serine, or glutamic acid) for growth. The fungus does not require an exogenous supply of vitamins in vitro.

Introduction

Orchids are generally infected with mycorrhizal fungi during some stage of their life cycle (Bernard, 1909; Harley, 1969). Germination of their seeds requires fungal infection and the fungi have been implicated in the supply of nutrients. The observation that seedlings of *Neottia nidus-avis* (Orchidaceae) were infected with fungal hyphae led to the assumption that the infection was required for germination (Bernard, 1899). Subsequent experiments using pure cultures of the fungus to infect and germinate orchid seeds proved this assumption to be correct (Bernard, 1899, 1909). These findings also served as a basis for the symbiotic method of commercial orchid seed germination (Ramsbottom, 1922).

Barely fifty years ago, asymbiotic germination of orchid seeds on a simple medium containing sucrose was achieved (Knudson, 1921, 1922). As a result of that and subsequent research, it is now generally believed that the action of the mycorrhizal fungus is to hydrolyze macromolecules and provide soluble sugars to the germinating seed (Arditti, 1967). Hydrolysates may be taken up directly by the orchid, transported into the plant by the fungus or obtained by the

digestion of intracellular hyphae (Burgeff, 1936; Ernst, Arditti & Healey, 1971; Harley, 1969; Smith, 1966, 1967).

Some of the nutritional requirements of selected orchid fungi have been determined (Burgeff, 1959; Downie, 1949; Hadley, 1969; Hijner & Arditti, 1973; Holländer, 1932; Mariat, 1951; Stephen & Fung, 1971). In several instances it has been shown that the fungi require vitamins or their precursors, amino acids or combinations of these. Such requirements suggest the possibility of nutritional exchanges between orchids and their mycorrhizal fungi. The present study was undertaken for the purpose of obtaining additional information concerning the specific growth requirements of *Rhizoctonia repens* M 32, an isolate from *Orchis militaris*.

Materials and methods

Culture media

The inorganic salts of (Knudson, 1946) plus micronutrients (Harrison & Arditti, 1970) were used as the basal medium (KC). As necessary, 2% sucrose (KCS); 2% glucose (KCG); sucrose and Difco Bacto-Peptone, each at 2% (KCSP), or 2% glucose plus amino acids were added to KC. Amino acids and glucose were reagent grade whereas sucrose was table quality. KCSP was sterilized by autoclaving. All other media were cold sterilized by filtration using $0.22\,\mu$ Millipore filters. Initial pH of all culture media following sterilization was 5.70 ± 0.01 unless otherwise indicated.

Inoculum

Cultures of the fungus *R. repens* M 32 were obtained from Dr. H. Kern (Eidgenössische Technische Hochschule, Zürich). They were maintained in Difco potato dextrose

broth or KCG plus 1.5% glycine, pH 5.70. Inocula were taken from 15 to 25 day old cultures. A 7 mm cork borer was used to prepare disks from mycelial mats to inoculate the experiments with peptone concentrations (fig. 1). The average dry weight per inoculum was 1.8 mg. When a wire loop was used in later experiments the average of inoculum dry weight was approximately 0.1 mg.

Culture conditions

Test tubes (25 mm × 190 mm) containing 25 ml of slanted media were used to culture the fungus during experiments. Temperature was constant at 24 °C. The cultures were maintained in dim light and 16 hour photoperiods.

Growth analysis

The fungus was harvested by filtration on pre-weighed filter paper, allowed to air-dry 4 days at room temperature and reweighed. Results are given in dry weight (DW) to the nearest mg and represent the averages of replicates.

Results

Fungus grown in KCSP averaged 115 mg per tube at 15 days and 215 mg after 25 days. When a loop was used the yield per culture was 107 mg and 203 mg respectively.

DW at 20 days was proportional to peptone concentration in KCS (fig. 1). Growth became proportional to concentration after 14 days. At 8 days the DW on all three peptone concentrations was nearly equal. Growth was limited in KC plus 2% peptone whereas the fungus did not grow on KCS (fig. 1).

There were two pH optima in KCSP, one at pH 5.65 to 6.10 and the other at 8.20 (fig. 2). Growth was reduced between pH 6.10 and 8.20. There was no appreciable growth below pH 4.05.

Growth on media containing glycine, glutamic acid or aspartic acid, whether alone or in combination with other amino acids was at least 66% of the control (KCSP) after 25 days (fig. 3, groups 5–15). The remaining amino acids present in peptone failed to support comparable growth (fig. 3, groups 1–4). Growth rate tended to be faster in the groups which contained only one or two amino acids and was especially rapid in the presence of glutamic acid alone (fig. 3, group 6). A medium containing all 15 amino acids present in peptone and at the same concentrations as in the control solution did support growth which was essentially equivalent to the control (fig. 3, groups 15 & 16; table 1).

The initial concentrations of amino acids in the defined
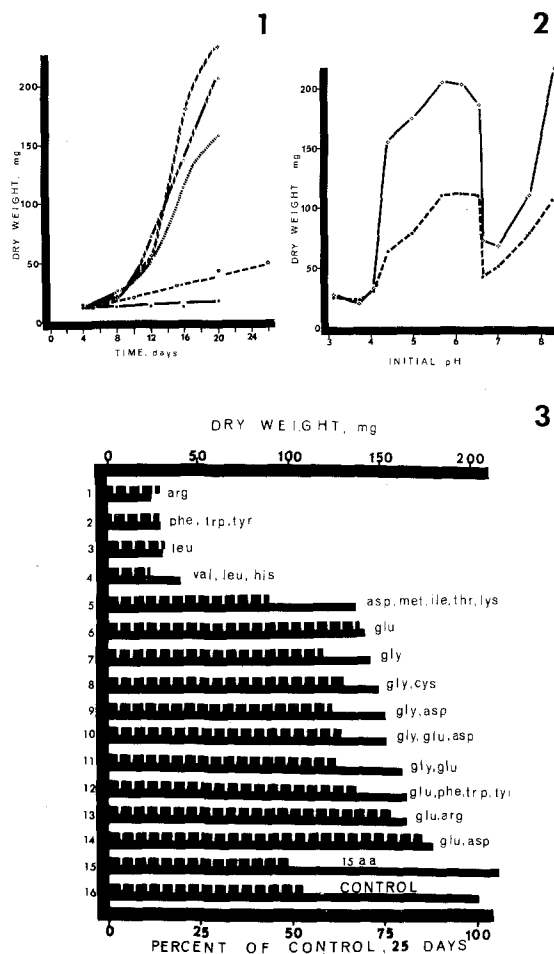
Figs. 1–3. Growth of *Rhizoctonia repens* M 32 in selected media. 1. Effects of peptone on growth in Knudson C culture medium. Explanation of symbols: slashed line and spiked open circles, 3% peptone; slashed line and open triangles, 2% peptone; dashed line and open circles, 1% peptone; dashed line and open asterisks, 2% peptone (minus sucrose from basal medium); solid line and solid squares, Knudson C (basal medium). 2. Growth as affected by the initial pH of the medium (Knudson C plus 2% peptone). Explanation of symbols: dashed line and solid circles, 15 days growth; solid line and spiked circles, 27 days growth. 3. Growth on peptone or amino acids as supplements to the basal medium (Knudson C). Amino acids were used at the concentrations present in 2% peptone solution except for leucine which when employed alone was present at 22.6 mM. Explanation of symbols: dashed line, 15 days growth; solid line, 25 days growth.

medium were based on those of a 2% peptone solution. Subsequently glycine and glutamic acid, each at two concentrations, were tested to compare their effects. Serine, which is not present in peptone was also used to determine

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Table 1

Typical amino acid analysis of difco bacto-peptone

Amino acid	Percent	Molarity in 2% peptone solution* × 10⁻³
Arginine	8.0	9.17
Aspartic acid	6.0	8.85
Cystine	0.22	3.63
Glutamic acid	11.0	14.9
Glycine	23.0	61.2
Histidine	1.0	ꞏ1.32
Isoleucine	2.0	3.05
Leucine	3.5	5.35
Lysine	4.5	5.88
Methionine	1.0	1.34
Phenylalanine	2.5	2.78
Threonine	1.5	2.68
Tryptophan	0.42	0.39
Tyrosine	1.0	2.54
Valine	3.0	5.45

* Calculated on anhydrous basis.

if its suitability paralleled its structural and metabolic similarity to glycine (table 2).

Two growth indicies, one based on molarity and the other on equal numbers of carbon atoms supplied were used to compare the amino acids (table 2). Aspartic acid (14.9 mM) supported best growth. It was followed by glycine (31.0 mM), serine (31.0 mM) and glutamic acid (14.9 mM) on the basis of equal carbon supplied. The order is slightly altered when the comparisons are based on equal molarity, but these amino acids remain superior to the others (table 2). Glycine (31.0 mM) was more effective than the higher concentration (61.2 mM). Glutamic acid at 61.2 mM was substantially less effective than at the lower concentration (14.9 mM) on both indicies (table 2).

Discussion

Although we refer to our fungus as *R. repens* M 32 it may prove to be a different taxon (J. H. Warcup, Waite Agricultural Research Institute. Adelaide. South Australia: personal communication). Several *Rhizoctonia* fungi from orchids have been found to be species of *Ceratobasidium*, *Sebacina*, *Thanatephorus* and *Tulasnella* (Warcup & Talbot, 1967). Isolates referred to as *R. repens* by Bernard may be *Tulasnella calospora*, possibly a ubiquitous orchid endophyte (Hadley, 1970). At least one strain of *T. calospora* requires yeast extract for growth (Hadley, 1970), whereas *R. repens* M 32 did not exhibit such a need. However nutritional requirements of orchid fungi should not be taken to indicate systematic relationships since they may vary from strain to strain (Hijner & Arditti, 1973; Mariat, 1948, 1951; Stephen & Fung, 1971; Vermeulen, 1947). It is simply necessary to wait for the final identification of *R. repens* M 32 by a systematist.

Obviously, peptone contains substances which are required by *R. repens* M 32 (fig. 1). Growth in a synthetic medium containing the 15 amino acids present in peptone suggests that vitamins and other factors were not required by *R. repens* M32 (fig. 3, groups 15 & 16). The vigorous growth of our fungus after serial transfer through at least three subcultures in vitamin-free medium also supported this view.

Despite considerable research on the nitrogen nutrition of fungi, generalizations regarding the suitability of various sources are not possible. Attempts have been made to classify fungi in accordance with their ability to utilize different forms of nitrogen (Robbins, 1937; Steinberg, 1950). However, in some cases the ability to utilize inorganic nitrogen is pH dependent (Hacskaylo, Lilly & Barnett,

Table 2

Growth of rhizoctonia repens M 32 on selected amino acids

Amino Acid	n*	mM	Dry weight, mg at 25 days	DW/mM	DW × mM/n
Aspartic acid	4	14.9	152	10.2	2.6
Glycine	2	31.0	158	5.1	2.5
Serine	3	31.0	183	5.9	2.0
Glutamic acid	5	14.9	141	9.5	1.9
Glycine	2	61.2	144	2.4	1.2
Glutamic acid	5	61.2	149	2.4	0.5
Arginine	6	9.2	26	2.8	0.5
Leucine	6	22.6	31	1.4	0.2

* Number of carbon atoms per molecule of amino acid

1954). Therefore, it may be misleading to classify our fungus into a particular group because of such influences.

Nitrate and ammonium present in the basal medium did not support growth in the pH range used (fig. 1). Amino acids could be a potential source of reduced nitrogen. They may be especially important if the organism was unable to use reduced inorganic forms. Limited information is available on the mode of amino acid utilization. The extent to which they are utilized as respiratory substrates versus their role as sources of an appropriate form of nitrogen is not well known.

Of the amino acids tested, only aspartic acid, glycine, serine and glutamic acid at certain concentrations supported good growth (fig. 3; table 2). Aspartic acid (14.9 mM) ranked first on a molarity basis (table 2) and when compared on total amino acid carbon was equal to glycine (31.0 mM). It easily enters reversible transaminations with α-ketoglutaric acid producing oxaloacetic and glutamic acids. Deamination yields fumaric acid (a Krebs cycle intermediate) and ammonia. In either situation, aspartic acid may represent a convenient source of amino nitrogen and/or serve as a precursor for other compounds.

On a molar basis glycine (31.0 mM), the smallest amino acid, ranked fourth (table 2). Its primary function may be to supply reduced nitrogen. If this were the case, growth on glycine would be slower due to the fact that it provided small carbon fragments. Deamination produces glyoxylic acid which may be oxidized in the Krebs cycle.

Equimolar concentrations of serine and glycine supported similar growth (table 2). In general biosynthetic schemes serine is considered to be a precursor of glycine. Deamination of serine yields pyruvic acid, which can enter the Krebs cycle, and ammonia.

Glutamic acid is the principal amino acid of transamination reactions. Most investigators have found it to be among the amino acids most readily utilized by fungi (Cochrane, 1958; Lilly & Barnett, 1951). Two *Rhizoctonia* endophytes of the orchid *Arundina chinesis* have been shown to use glutamic acid as a nitrogen source (Stephen & Fung, 1971). Three species of *Pestalotia* were found to utilize glutamic acid and proline as the best sources of nitrogen (Mitra & Tandon, 1970). Nitrogen sources supporting the best growth in the genus *Linderina* (Zygomycetes) include dl-glutamic acid, aspartic acid and asparagine (Stephen & Chan, 1970). The suitability of glutamic acid was attributed to its importance in transamination reactions. It represents a convenient source of amino nitrogen and also contains a relatively large carbon skeleton.

Failure of leucine (22.6 mM) to support growth may reflect the inability of the fungus to deaminate it. Such a situation has been observed in *Streptomyces venezuela* (Gottlieb & Ciferri, 1956). The same may be true regarding the other amino acids which did not support growth. Additional factors may include uptake by the fungus, charge on the amino acid molecules, pH of the medium, absence of enzymes required for reactions involving these compounds, or combinations of these and other aspects.

It is noteworthy that the amino acids which supported the best growth (fig. 3; table 2) are close to glycolysis and the Krebs cycle. All appear to be capable of supplying nitrogen either through transamination or deamination. The extent to which the remaining carbon skeletons influence growth of the fungus is not clear.

Little is known about the specific nutritional requirements of orchid endophytes in vitro. General information on fungal nutrition may be of considerable value in understanding mycorrhizae where nutritional balance is believed to be delicate (Harvais & Hadley, 1967a).

R. repens M 32 is an isolate from *O. militaris* (the soldier orchid). Of the many so called typical orchid endophytes which include strains of *R. repens* few have been isolated from soil (Harvais & Hadley, 1967b). Some isolates have been reported to lose viability after extended periods in culture, presumably due to the depletion of required factors (Harvais & Hadley, 1967a). Our findings suggest that this is not the case with this *R. repens* M 32. Suitable amino acids in a glucose containing basal medium are sufficient to maintain its growth. However it is not clear at this time whether our fungus has retained the capacity to establish a mycorrhizal relationship with orchids. Another point in need of further investigation is the role played by amino acids (and other compounds) in attracting fungi to orchid seeds.

Summary

Rhizoctonia repens M 32, an orchid mycorrhizal fungus, required aspartic acid, glycine, serine or glutamic acid in addition to glucose or sucrose. Nitrate and ammonium in the basal medium did not satisfy the nitrogen requirements at the pH used. Growth of *R. repens* M 32 in a synthetic medium containing 15 amino acids paralleled that in an undefined peptone containing one. This isolate did not require an exogenous supply of vitamins. The relationship of *R. repens* M 32 with *Orchis militaris* is discussed in terms of mycorrhiza and fungal metabolism.

Acknowledgements

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