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Studies of physiological effects of fungicides on fungi are critical for understanding modes of action and mechanism of resistance; however, studies are difficult to perform on filamentous fungi because of the inability to produce homogeneous a biological system using a mass of mycelium. Moreover, the presence of rigid cell wall can limit uptake of chemicals. An alternative approach is to produce protoplasts. A method for isolating protoplasts from common wood-degrading fungi Phanerochate chrysosporium, Postia placenta, Gloeophyllum trabeum, and Trametes versicolor with Novozyme 234 is descibed. Generally, protoplasts were more readily isolated from younger hyphae, while regeneration was better with older hyphae.

Fungicidal action was evaluated against Trametes versicolor and Postia placenta using both protoplasts and cell fragments. Generally, similar inhibitory effects were observed with cell viability, glucose utilization, cell respiration, permeability, and

copper absorption bioassays. Although protoplasts showed a higher sensitivity to toxicants, this sensitivity was limited compared to that of plant protoplasts. The loss of vulnerable, senescing cells, alteration of some properties of protoplasts during or after lytic enzyme digestion, or biocide absorption by non-viable protoplasts might result in reduced sensitivity of protoplasts to chemicals. The results suggests that further studies are required to delineate more fully the optimum conditions for physiological activity of protoplasts before the full effectivness of the system can be exploited for biocidal action research.

Isolation of Protoplasts from Selectd Wood Degrading Fungi and Their Useds in Studying Fungicidal Action

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ISOLATION OF PROTOPLASTS FROM SELECTED WOOD DEGRADING FUNGI AND THEIR USES IN STUDYING FUNGICIDAL ACTION

1. General Introduction

Studies of physiological effects of biocides on fungi are fundamental for understanding modes of action and mechanisms of resistance. This knowledge can be used to guide the design of new fungicides. Ever-increasing environmental pressures have created an intensive need to develop safer, more selective fungicides. While many agricultural fungicides are well studied , the chemicals used to protect wood have received far less attention. This lack of attention reflects the wide array of potential target organisms as well as the long time periods required for many tests.

Wood decay fungi usually exist as filamentous hyphae and are generally classified by the type of wood decay they cause into white, brown and soft rot. These fungi share a characteristic typical of most fungi- the presence of a rigid cell wall. Although important structurally (Farkas, 1985), the cell wall interferes with studies of interactions between fungicides and fungi (Theuvenet, 1980; Gadd et al., 1984), acting as a barrier to limit uptake of toxicants. In addition, most physiological activity occurs near the growing tip area of fungal hyphae, where enzymes are secreted and wood-decomposition products are reabsorbed into the hyphae. As a result, studies of the mycelial phase of the fungus require large amounts of hyphal mass for observation of effects on a relatively few cells.

An alternative approach to the use of hyphae for physiological studies is to produce

protoplasts by removing fungal cell walls (Collings et al., 1989). Protoplasts have been widely used for inserting desirable genetic characteristics into fungi and plants and for studies of fungal morphology, but they can also be useful for monitoring the effects of toxicants on cells (Gadd and White, 1985; Gadd et al., 1987; Kihn et al, 1987). In addition, protoplasts are easily disrupted by osmotic lysis, making it easier to prepare subcellular or cell free materials which are usually necessary for studies of biocidal action.

Although methods have been developed for isolating protoplasts from edible basidiomycetes (Kitamoto et al., 1984,1988; Yanagi et al., 1990; Toyomasu et al., 1986; Sudo and Higaki, 1988; Eguchi et al., 1990; Tamai et al., 1990), heartrot fungi (Trojanowski et al., 1984) and an array of fungi imperfecti (Sivan et al., 1990), there are few protocols available for producing protoplasts from common wood decay fungi. The objectives of this study were to:

* identify the optimal experimental conditions for the isolation of protoplasts from three important species of wood degrading fungi.
* assess the physiological effects of selected fungicides on isolated protoplasts.

2. Literature Review

2.1. Isolation and Regeneration of protoplasts

2.1.1. Introduction

Fungal protoplast production and regeneration have been subject to extensive study because of their potential uses for genetic, biochemical and morphological processes. Protoplasts have been isolated from almost every major taxonomic group, mostly by enzymatic digestion (Peberdy, 1979). One of the most important considerations in protoplast production is to maximize yield, while retaining protoplast viability. Therefore, the review will be directed to factors which influence protoplast production, emphasizing the basidiomycetes since many important species of wood decay fungi belong to this group.

2.1.2. Chemical Composition, Organization and Function of Fungal Cell Wall

Chemical composition and properties of the fungal cell wall are briefly discussed because they can help understand the process of protoplast isolation by enzymatic digestion. More extensive information on this subject is available elsewhere (Bartnicki-Garcia, 1968; Phaff, 1977; Farkas, 1985)

The fungal cell wall fulfills several functions connected with the interaction of the cell with its environment (Farkas, 1985). These major roles are: (1) the formation of rigid mechanical barrier on the surface of the protoplast, which determines the shape of the cell, (2) acting as an osmotic protector to the protoplast, preventing it from uncontrolled expansion and bursting in the normally hypotonic environment, (3) acting as the site of various extracellular enzymes engaged in the exchange of nutrients and products of metabolism as well as in hydrolysis of cell wall components during cell wall expansion (Lampen, 1968; Phaff, 1977). The multifunctional nature of the cell wall is undoubtedly a reflection of its particular chemical composition and of the spatial organization of its individual components.

Fungal cell walls are composed of homo- and heteropolysaccharides, proteins, Table 2.1. Chemical composition of isolated cell walls from S.commune and A. nidulans

component	S. commune [1] (% of wall dry weight)	A.nidulans [2] (% of wall dry weight)
Glucose	67.6	39.0
Mannose	3.4	4.0
Galactose	0.2	9.5
Galactosamine	2.2	2.3
Glucosamine	-	13.5
Protein	-	3.5

[1] Data from De Vries and Wessels (1975)

[2] Data from Bainbridge et al.(1979)

glycoproteins and variable amounts of minor constituents such as lipids and melanins (Barticki-Garcia, 1968; Rosenberger, 1976). Table 1 gives typical analysis of cell walls from Schizophyllum commune, a wood decay fungus, and a mold, Aspergillus nidulans.

In most fungi, polysaccharides represent over 75% of the cell wall dry weight. According to their function, they may be classified into two groups: skeletal polysaccharides and matrix polysaccharides. The first group of polysaccharides are represented by water-insoluble, highly crystalline polysaccharides such as glucan, cellulose, and chitin. These polysaccharides give the wall mechanical rigidity and maintain its shape. The polysaccharides of the wall matrix are usually amorphous, or slightly crystalline, homo-and heteropolymers which often occur in chemical association with proteins. These polysaccharides fill the space between skeletal polysaccharide microfibrils, serving as a cementing substance.

The spatial arrangement of the cell wall components are one of the most characteristic ultrastructure features of fungal cell walls. Skeletal, microfibrillar wall components, such as glucan, chitin or cellulose, are embedded in an amorphous polysaccharide and glycoprotein matrix. The outer surface of the wall is usually smooth or slightly reticular, whereas the skeletal polysaccharide microfibrils are more prominent on the inner surface of the wall. For example, the innermost cell wall layer of S. commune, is composed of chitin microfibils embedded in R-glucan, and the outermost layer of cell wall mucilage is composed of a mixed, alkali-soluble ß-1,3 and ß-1,6-glucan (Van der Valk et al., 1977).

Composition and organization of cell walls of individual fungi species have profound and subtle influences on the isolation of protoplasts (Peberdy, 1972). For example, Sietsama et al (1968) showed that cellulase and laminarinase were essential for protoplast production from Pythium sp, while chitinases and S-glucanase (α-1, 3-glucanase) were necessary for the isolation of protoplasts of S. commune. R-glucanase (ß-glucanase) was also required for S.commune, but could be derived endogenously from the fungus. The requirement for cellulase or chitinase for protoplast production from two organisms reflects the major differences in composition of their cell walls. The variation in cell wall composition between and within species may also have significant effects on the digestion efficiency of lytic enzymes. The effects of composition and structure on protoplast isolation will be addressed in a later section.

2.1.3. Lytic Enzymes for the Isolation of Fungal Protoplasts

Interest in lytic enzymes was derived from a desire to understand the chemical nature and ultrastructure of the fungal cell wall. Enzymes could be used to selectively remove the constituents of the wall by a technique known as "enzyme dissection".

The first report on the use of lytic enzyme against fungi was described the activities of snail enzyme against chitin and " yeast glucan or cellulose". Extensive studies on the sources of lytic enzymes and characteristics of those enzymes have been carried out since then and several lytic enzyme sources have been commercially produced (Hamlyn et al., 1981).

Eddy and Williamon (1957) reported the successful application of snail (Helix pomatia) digestive juices as a lytic enzyme to produce protoplasts from certain strains of Saccharomyces cerevisiae and S. carlsbergensis. Holden and Tracey (1950) detected at least 30 enzymes in snail gastric juice, of which 20 were polysaccharases. One of the few detailed analyses of these enzymes was carried out by Anderson and Millbank (1966). This enzyme is now commercially available as a helicase, gluculase, glucuronidase, and sulfatase and has been used extensively for isolating protoplasts from various types of yeast and filamentous fungi (Peberdy and Gibson, 1971).

The exposure time required for optimal protoplast release, using snail enzyme, varies from species to species. Protoplasts require 3-6 hr for release from Saccharomyces cerevisiae (Kreger at al., 1975) and 7-10 hr for Geotrichum candium (Sagara, 1969). Enzyme exposure period is an important consideration, since prolonged incubation may cause protoplasts to degenerate.

Enzyme exposure can be limited by providing supplemental enzymes. Thomas et al.(1979) reported that the addition of chitinase to snail enzyme increased the yield of protoplasts from Aspergillus niger and A. fumigatus. De Waard (1976) also noted that the mixtures of commercially available enzymes promoted protoplast release from sporidia of Ustilago maydis.

Although snail enzyme is effective for many species of fungi, especially for yeast, it is less effective for the isolation of protoplasts from basidiomycetes. De Vries at al (1972) reported that the lytic enzymes from Trichoderma harzianum produced favorable yields of protoplasts from many species of basidiomycetes including Collybia velutipes,

Coprinus lagopus, Lentinus triginus, Pleurotus corticatus, Pholiota aurivella, Hericium coralloides, Polyporus biennis, and Polystictus versicolor. This enzyme preparation also released protoplasts from ascomycetes such as Neurospora crassa, various Penicillium species, and A. nidulans. Their studies indicated that Helicase, a snail enzyme, lacks ß-1,3-glucanase (Kanetsuna et al.,1969; De Vries and Wessels, 1973a), although it contains at least 30 different hydrolases. However, this enzyme was present in the Trichoderma lytic enzyme system when the organism was grown on ß-1,3-glucan (Hasegawa and Nordin, 1969) or on the cell walls of S. commune (De Vries and Wessels, 1972). ß-1,3-glucanase is essential for protoplast release from S.commune and a large number of other fungi containing ß-1,3-glucans (Wessels et al., 1972). The presence of the enzyme may account for the effectiveness of the Trichoderma enzyme preparation in releasing protoplasts in these fungi. In the further studies, the same authors (1973 b) detected the R-glucanase, S-glucanase and chitinase in the enzyme preparation by using carboxymethyl cellulose and Sephadex G100 columns. Kitamoto et al.(1984) reported the factors influencing the lytic enzyme production from Trichoderma harzianum. Production of lytic enzymes from Trichoderma harzianum is greatly affected by substrates and carbon and nitrogen sources in the medium. Both ß-1,3-glucanase and chitinase activities increased 10-50 fold and cell wall lytic activity also increased 50-100 fold when a fraction of cell wall polysaccharides, mycelial powder or fruiting bodies of basidiomycetes was added to the culture medium. Nitrogen sources are also needed for the production of lytic enzymes, although no difference in the effects of organic and inorganic nitrogen sources on the productivity of lytic enzymes could be

distinguished.

A commercial enzyme preparation of T.harzianum (NOVOZYM 234) is effective for fungal protoplast isolation. Favorable yields of protoplasts have been produced from Pleurotus ostreatus (Ohmasa et al., 1987) and Phanerochate chrysosporium (Gold et al., 1983). Other important sources of lytic enzymes include Arthrobacter luteus (Kitamura et al., 1974: Doi et al., 1971) Oerskovia xanthineolytica (Scott and Schekman, 1980) , Bacillus circulans (Tanaka et al., 1965), Cytophaga johnsonii (Bacon et al.,1965) and Streptomyces satumaensis (Villanueva and Garcia- Acha, 1971). These enzyme preparations show different enzyme constituents and have been used to produce protoplasts from yeasts. Commercial products, Zymolyase 5000 and Zymolyase 6000, have also been used for protoplast isolation of certain species of basidiomycetes (Ohmasa et al., 1987).

2.1.4. Osmotic Stabilizers

An important functions of the fungal cell wall is to act as an osmotic stabilizer to the protoplast. The removal of cell wall by lytic enzyme will result in the exposure of protoplasts to an environment which has higher water potential, causing immediate lysis. This is especially critical for protoplast production unless the osmotic concentration is adjusted. As an effective osmotic stabilizer, a chemical should fulfill three requirements: (1) inable to penetrate into protoplasts at an appreciable rate; (2) non-toxic to isolated protoplasts; and (3) not inhibitory to lytic enzyme activity.

A wide range of osmotic stabilizers including inorganic salts, sugars, and sugar alcohols have been used to stabilize protoplasts released from fungi (Davis, 1985). The type and concentration of stabilizer may influence both protoplast yield and stability; these factors also depend on the organism. These factors are highlighted in a number of comparative studies of osmotic stabilizers with varying fungal species (Sietsma and de Bore, 1973; Peberdy et al., 1976). Some osmotic stabilizers such as mannitol, sorbitol, KCl and $MgSO_4$ are frequently employed. Mannitol, a sugar alcohol, is an excellent, metabolically inert osmotic stabilizer. Sorbitol, an isomer of mannitol, is less frequently used, although it is more soluble and less expensive. In some cases, sugar alcohols appear to inhibit lytic enzyme activity (Arnold and Garrison, 1979). Mannitol and sorbitol are also excellent for maintenance of protoplasts (Arnold and Garrison, 1979).

Some inorganic salts appear to be more useful for the protoplast isolation from filamentous fungi, while sugar alcohols are more effective for isolation of yeast protoplasts (Davis, 1985). Isolated protoplasts have a slightly reduced activity in the presence of certain salts, since salts are more likely than sugars to penetrate into the cells during long incubation periods (Evans at al., 1973). However, stimulatory effects of some inorganic salts on lytic enzymes have also been observed (Rodriguez Agurre et al., 1964; Thomas and Davis, 1980). The addition of $CaCl_2$ to the isolation medium, containing KCl, a stabilizer, greatly enhanced the release of protoplasts from A. niger and A. fumigatus (Thomas and Davis,1980). This effect was inconsistent with concentration, fungal isolate, and lytic enzyme employed, but was greatest when

chitinase was included in the lytic enzyme system.

Magnesium sulfate has promoted the release of highly vacuolated protoplasts from S. commune (De Vries and Vessels, 1972) and A. nidulans (Peberdy and Isaac, 1976), with the added advantage that these protoplasts can be easily isolated from mycelial debris as they float on the supernatant after centrifugation. However, protoplasts isolated from A. fumigatus in the presence of $MgSO_4$ were osmotically very fragile (Hearn et al., 1980).

The relative density of isolated protoplasts may be affected by different stabilizers. For example, S. commune protoplasts isolated in NaCl, KCl, Sorbitol, and mannitol can be sedimented under gravity, but remain in suspension in sucrose, indicating they have a density similar to that of the isolation medium (De Vries and Wessels, 1972).

The optimum concentration of stabilizers for protoplast isolation varies from species to species. For example, the concentration of rhamnose used for the isolation of protoplasts from Saccharomyces carlibergensis is 0.5 M (Eddy and Williamson, 1957), but concentrations as high as 2.0 M have been used for Saccharomyces mellis (Weinberg and Orton, 1965). Optimum protoplasts yields can generally be obtained using 0.5 M of mannitol or $MgSO_4$ for most species of basidiomycetes and yeasts (De Vries and Wessels, 1973 a and b; Yoshinika et al., 1986).

2.1.5. Age of Cultures

The influence of culture age on protoplast isolation has been well documented. In

general, protoplast yield is much higher from cultures in the early exponential growth phase than from those in stationary or late exponential growth phases (Benitez et al.,1975; Peberdy et al., 1976). Schaerncke et al. (1977) reported a 75% decrease in protoplast yield from cultures in stationary growth phases or from starved cells from various strains of S. cerevisae and other yeasts. However, great decreases in protoplast yield were noted when cultures in late exponential growth phase or stationary phase for basidiomycetes were employed (Ohmasa et al., 1987; Eguchi et al., 1990). Protoplast yield was $8x10^6$/g fresh weight mycelium with 2 day old cultures of Grifola frondosa FMC321, while few protoplasts were isolated from 4 day old cultures of the same fungus (Ohmasa et al., 1987).

It is unclear why mycelium yields far fewer protoplasts in the late exponential growth phase or stationary phases. A buildup of melanin in the later stages of growth in A. nidulans may mask the action of the lytic enzymes on the mycelial wall (Carter and Bull, 1969; Bull, 1970a,b). Bartnicki-Garcia and Lippman (1972) have suggested that the high concentrations of endogenous wall-building enzymes during the exponential growth phase may complement the effect of the exogenous lytic enzymes on the cell wall, giving greater protoplast yields during this phase of growth. Changes in wall structure of S.commune may also be responsible for reduced protoplast yields (De Vries and Vessels, 1972) and this would be in agreement with results of studies on the cell walls of Geotrichum candidum (Sagara, 1969).

2.1.6. Culture Conditions

Culture conditions including medium constituents, type and concentration of buffer, aeration may all affect fungal protoplast isolation. Experiments with A. nidulans have shown that protoplast yields are higher from mycelium growing on a glucose-salts medium than mycelium growing on yeast extracts (Peberdy, 1976). Musilkova and Fencl (1968) found that A. niger grown on mineral medium supplemented with glucose and asparagine released more protoplasts per milligram of mycelial dry weight than mycelium grown on the same medium supplemented with malt extract or on malt extract alone. Yoshinika and Yanage (1986) found that both protoplast yield and the efficiency of protoplast regeneration could be greatly enhanced when Pleurotus ostreatus mycelium was grown on the MYPG medium supplemented with sulfate pulp waste which included monosaccharides, polysaccharides and sulfated lignin carbohydrate complexes. The yield of A.nidulans protoplasts can be greatly increased by lowering the phosphate concentration and introducing a chelating agent to the medium (Van den Broek et al.,1979).

The effects of culture conditions are poorly understood, but nutritional status may affect the arrangement or quantities of various components of cell walls which, in turn, affected protoplast production (Bartnicki-Garcia and Nickerson, 1962).

2.1.7. Other Factors Influencing Protoplast Release

Temperature and pH both have significant effects on the activity of the lytic enzyme system. Thomas (1981) reported that the optimum temperatures for protoplast release from A. niger and A. fumigatus were 30 and 35 °C, respectively. Higher temperatures resulted in a significant decrease in protoplast yields, however, protoplasts isolated from A. niger at 50 °C were stable for up to 18 hr, suggesting that reduced yield of protoplasts at higher temperature was due to inactivation of the lytic enzymes. Unfavorably high temperatures may cause agglutination of certain organelles in isolated protoplasts and may render protoplasts unsuitable for metabolic studies, while low temperature may affect membrane stability (Kovac and Subik, 1970). The optimum temperature for protoplast isolation from basidiomycetes falls in the range of 30 to 35 °C (Davis, 1975).

The pH of the incubation medium may also affect protoplast yield. Protoplast production from Penicillium chrysogenum occured between 4.0 to 8.0 pH, but optimum release occurred at 5.5 pH (Eyssen, 1977). Little evidence of mycelial digestion was observed at pH values below 4.0 or above 8.0. These results probably reflect enzyme inactivation at pH extremes.

The choice of buffer in the incubation medium can also affect protoplast yield from basidiomycetes. Phosphate buffers can interfere with osmotic stabilizers (De Vries and Wessels, 1972) and enzyme activity (Gasco et al., 1965). Yanagi et al.(1985) also reported that few protoplasts were produced when an acetic acid-NaOH buffer was

employed. The buffer most widely used for protoplast isolation from basidiomycetes was 50 mM maleic-NaOH with pH 5.0-6.0 (Yanagi, 1985; Vries and Wessels, 1973 a).

2.2. Protoplast Regeneration

2.2.1. Introduction

The process of restoring normal cell walls of isolated protoplasts is referred to as protoplast regeneration. Protoplast regeneration can be used as a measure of protoplast repair capacity and cell health. In this review, only factors influencing protoplast regeneration will be discussed. Comprehensive reviews on the mechanism of protoplast regeneration are available elsewhere (Wessels et al.1976; Peberdy, 1979; Necas and Svoboda, 1981).

2.2.2. Regeneration Capacity of the Fungal Protoplasts

Generally, the protoplasts are characterized by loss of only the cell wall. In uninucleate fungi, the protoplast is equal to the essential content of its intact cell or its multiple if it results from a dividing cell (Ottolenghi,1966; Kopecka,et al., 1974). Mycelial fungi, however, produce protoplasts of more heterogeneous structure, with varying numbers of nuclei and other cell organelles, arising from the uneven distribution of these organelles in the original hyphae. For this reason, protoplasts isolated from

fungal spores are often used to produce more homogeneous systems for the studies of biochemical and genetic processes. The release of protoplasts from different parts of the hyphae also contributes to heterogeneity (Gibson and Peberdy, 1972; De Vries and Wessels, 1975). This implies that in terms of structure, the protoplast is not always identical. Moreover, the isolation of protoplasts brings about a number of structural and functional alterations, which later must be repaired. These may include changes in ultrastructure, distribution of membrane organelles (Havelkova, 1966; Mannocha, 1968), and changes in the structure of the plasma membrane (Necas et al., 1969). In addition, changes in the architecture of the cytoskeletal system and the loss of substances in the periplasmic space may occur.

It is believed that the protoplasts of any fungal species which contains a nucleus is capable of repairing cell damage, including regeneration of the missing cell wall. The cell can also repair all other alterations, producing a complete and functional cell. Unfortunately, repair potential may not be present in the every protoplast of the population due to damage during the process of isolation or other the effects of environmental conditions in the process of regeneration. Isolation conditions including the time length and the temperature of incubation, sources of lytic enzymes, and types of stabilizers and buffers can all affect protoplast regeneration (Necas et al., 1969).

2.2.3. Environmental Factors Influencing Protoplast Regeneration

Sugar alcohols are generally the most suitable osmotic stabilizers for protoplast

regeneration (Arnold and Garrison, 1979). Inorganic salts are effective for protoplast isolation, but they are ineffective for protoplast regeneration. For example, Ohmasa et al.(1987) examined the effects of sucrose, mannitol, sorbitol, glucose, $MgSO_4$ and $(NH4)_2S0_4$ as osmotic stabilizers on protoplast regeneration of Coprinus cinereus, and found that all were effective for colony formation except $(NH4)_2SO_4$ which inhibited the protoplast regeneration. The osmotic concentration optimal for colony formation was usually 0.5-0.6 M for mannitol, sucrose or $MgSO_4$.

Liquid and solid media have been widely used for protoplast regeneration; however, enzymes secreted into regenerating cell wall were retained at the protoplast surface in solid medium and lost into liquid medium reducing regeneration (Roland and Part, 1973). Conversely, Ohmasa et al (1987) reported that the preculture of protoplasts in liquid medium improved the regeneration efficiency.

Since regeneration is the process of cell wall restortion by protoplasts, the addition of cell wall components such as N-acetylglucosamine, a repeating unit of chitin, into the medium may improve the efficiency of regeneration (Ohmasa et al., 1987). Sietsma et al.(1973) reported that the addition of 1% glucose into medium increased the efficiency of protoplast regeneration by about 3 times for Pythium PRL2142. Glucose is essential for cellular energy metabolism and synthesis, but may also serve as osmotic stabilizer. Nitrogen sources, including asparagine, yeast extract and peptone, were not necessary but stimulated the process of regeneration considerably. Many workers have found that gelatin, agar, agarose or polyethylene enhanced the efficiency of regeneration (Necas, 1962; Svoboda and Piedra, 1983;). For example, regeneration improved 2-3 fold with

the addition of low temperature agarose (Eguchi et al. 1990). The incidence of the formation of normally shaped hyphae was much higher for S. commune in the medium solidified with gelatin.

Temperature and pH of the medium can have significant effects on the efficiency of protoplast regeneration. Higher and lower temperatures may affect the stability of cell membrane, while more acidic or basic pH may affect the activity of the cell wall synthetase system. The optimal protoplast regeneration with basidiomycetes has been obtained from 25-30 °C and pH 4.5 -6.5 (Ohmasa et al. 1987; Eguchi et al., 1990).

2.2.4. Conclusion

There are many factors influencing fungal protoplast isolation and regeneration including the medium and growth conditions of the organism, the physiological age and strain of organism, the lytic enzyme system, osmotic stabilizers, and incubation time and conditions. Owing to the variations in chemistry and structure within fungi cell walls, it is virtually imposible to use identical techniques and conditions for protoplast isolation from different species. Therefore, the optimal conditions need to be determined for each species and strain.

2.3. The Uses of Fungal Protoplasts for Studying Biocidal Action

2.3.1. Introduction

The isolation of fungal protoplasts has provided a novel approach for studying various fungal properties. Fungal protoplasts have been used for the studies of cell wall properties and biosynthesis (Moore and Peberdy, 1976; Isaac, 1978); properties and functions of the cytoplasmic membrane, external enzyme secretion (Boulton, 1965; Andres and Peberdy, 1974); biomacromolecular synthesis and localization of enzyme activities (Zelcer and Galun,1976; Tonino and Steyn-parve, 1963), and uptake and transport of substances (Elorza et al., 1969; Cowie and Walton, 1956).

Generally, the removal of cell walls from the membrane can help clarify the roles of cell wall and membrane components, improve the understanding of the processes of enzyme secretion, and localize the activities of some metabolically important enzymes. The removal of cell wall, which results in full exposure of cell envelope to environment, improves uptake and transport of substances, an important characteristic for studying fungicidal action, since uptake and transportation of toxic substances may be a prerequisite for toxicity. The absence of a cell wall also permits rapid monitoring of cell responses to manipulation of environmental factors and provides a homogeneous system for studying filamentous fungi, a difficult task with mycelium. Isolation and regeneration can provide considerable information on cell wall structure and synthesis under a variety of stresses. Finally, protoplasts are very useful for preparing subcellular and cell-free

substances. Although fungal protoplasts have been widely used for studying the biochemistry of fungi, this potential has not been well exploited in studying fungicidal action.

2.3.2. Use of Protoplasts for Studying Chemical Sensitivity

Chemical sensitivities of plant protoplasts have been extensively examined (Siegel, 1977; Earle, 1978). Generally, plant protoplasts show much higher phytotoxicity than callus cells or intact cells (Breiman and Galun, 1981). With this advantage, plant protoplasts have been used for screening and isolation of phytotoxins (Matern et al., 1978).

Although fungal protoplasts may not show higher sensitivity than intact cells under some situations, they can still be used to elucidate movement of toxicant and the roles of cell wall and membrane in toxicant resistance. Gadd and White (1984) investigated the relative distribution of copper by cell wall absorption and intracellular transport using whole cells and protoplasts of wild strain and copper-resistant mutants of Saccharomyces cerevisiae. The removal of cell wall did not alter the kinetics of uptake or significantly increase the chemical sensitivity. The decreased uptake in a copper-resistant mutant, reflected a real change in membrane structure and transport properties. Interestingly, the chemical sensitivity of protoplasts decreased compared with intact cells. This effect was attributed to the loss of vulnerable cells, such as small buds and senescing cells during the processes of cell wall digestion and protoplast purification.

Although basic properties of fungal protoplasts are believed to be the same as those found in intact cells, some property alterations during isolation are possible. The same authors investigated heavy metal ion uptake by intact cells and protoplasts from yeast-like cells and chlamydospores of Aureobasidium pullulans to clarify the mechanism of resistance. Chlamydospores were more tolerant to heavy metal ions than hyaline hyphae or yeast-like cells. The data confirmed that the tolerance property of chlamydospores was due to their thicker cell wall, since the protoplasts of yeast-like cells and chlamydospores showed a similar sensitivity to Cu, Zn and Cd ions. At the same time, the higher sensitivity of protoplasts indicated that the cell walls of yeast-like cells and chlamydospores play an important role in toxin resistance. Additional advantage of protoplasts was these consistent round shape which enabled easy measurement of volume and surface area. These values were used to produce more meaningful meaningful influx/afflux rates of chemical movement in terms of surface area. In the absence of the cell wall, light microscopic studies on cellular compartmentalization of ions, e.g., within vacuoles, were also more easily performed.

Theuvenet and Bindels (1980) also explored the feasibility of using yeast protoplasts to study the effects of toxicants on ion transport properties of the plasma membrane. They found that the antibiotic, Dio-9, reversed the metabolic proton extrusion into a net proton influx at least 50 fold higher after enzymatic removal of the yeast cell wall. Lampen (1968) investigated the effects of nystatin, an N-acetylcandicidin, on leakage of intracellular constituents from sensitive yeast cells and their protoplasts. Cellular constituents such as potassium ions, amino acids and nucleotides provide a measure of

cell membrane function disturbance (Lambert and Hammond, 1973). Sensitive yeast cells bound the antibiotic at 30 °C but not 0 °C. However, protoplasts could bind at either 0 °C and 30 °C and protoplasts took up the antibiotic very rapidly at 0°C. Many authors have also used bacterial protoplasts to demonstrate the effects of biocides on cell membrane function (Lambert and Smith, 1976).

In filamentous fungi, protoplasts also provide a more homogeneous experimental system for studying uptake and transport of toxic substances. The preparation of a homogeneous suspension is difficult with mycelium and there may be difficulties in removing free ions trapped in the interhyphal spaces. However, it is not difficult to produce a homogeneous system with protoplasts. Gadd (1985) demonstrated the energy-dependent copper influx and the influence of pH on toxicity using the protoplasts of Penicillium ochro-chloron.

2.3.3. Use of Protoplasts For Studying Inhibtion of Cell Wall Synthesis

Fungal protoplasts provide an excellent experimental system for studying cell wall synthesis. In fact, considerable information on cell wall synthesis has been accumulated using these systems (Cabib et al., 1974; Isaac, 1978). Protoplasts can also be used to study the effects of selective inhibitors of cell wall synthesis. Selective inhibitors of chitin synthesis may provide environmentally-acceptable fungicides (Jonson, 1986); however, studies of this aspect are limited (Sietsma and Wessels 1988; Elorza et al.,1987), and are primarily oriented towards understanding the mechanisms of cell wall

synthesis.

Sietsma and Wessels (1988) investigated the effects of 2-deoxyglucose and Polyoxin D on the cell wall synthesis of S. commune using protoplasts. They found that 2-deoxyglucose inhibited the synthesis of the alkali-soluble α-1,3-glucan, but the protoplasts were still able to generate hyphae. However, Polyoxin D inhibited the formation of chitin or alkali-insoluble wall glucan to prevent hyphal morphogenesis. Elorza et al.(1987) also reported an inhibitory effect of nikkomycin on synthesis of chitin by Candida albicans protoplasts.

2.3.4. Conclusion

The fungal cell wall represents a physical barrier, and many problems with studies of fungicidal action originate from its presence. As an alternative approach to the use of highly integrated hyphae, protoplasts may provide a novel system for studying fungicidal action. Protoplasts may have increased chemical sensitivity and enhanced toxicant transport in a relatively homogeneous system. They can also be used for preparing cell free and subcellular material.

SECTION I

3. Isolation of Fungal Protoplasts From Selected Wood-Degrading fungi

3.0. Introduction

In this section, protoplast production from several economically important species of wood degrading fungi was investigated. Attempts were made to identify factors influencing protoplast formation in order to maximize protoplast yields. Efforts were also made to clarify conditions for protoplast regeneration, since the major limitation in protoplast application was the availability of viable protoplasts in sufficient quantities.

3.1. Material and Methods

3.1.1. Fungi Species Tested

Four wood-degrading fungi were selected for the evaluation of protoplast isolation (Table 3.1). Phanerochaete chrysosporium, although not an important wood-degrading fungus in natural systems, was included for comparative purposes, as it has been the subject of intensive investigation for its lignin-degradation capabilities and for protoplast generation (Gold et al., 1983). All fungi were maintained on potato dextrose agar prior to use.

Table 3.1. Fungi evaluated for protoplast isolation

fungi species and sources	decay type
Gloeophyllum trabeum (Pers.:Fr.)Murr. (Madison 617)	Brown rot
Postia placenta (Fr.)M.Lars. and Lomb. (Madison FP94267R)	Brown rot
Phanerochaete chrysosporium Burds.(BKMF-1767)	White rot
Trametes versicolor (Fr.:Fr)Pilat (Madison R105)	White rot

3.1.2. Growth Media and Inoculation of cultures

Fungi were maintained in a medium containing: 10.0 g glucose, 10.0 g malt extract, and 1.0 g yeast extract per liter distilled water. Cultures were started by adding small plugs (3-mm diameter) cut from the actively growing edge of each culture to 50 ml of nutrient medium. The flasks were incubated 2, 4, 6, 8, 12, 14, 16, 18 days at 28 °C for the determination of growth curves and protoplast isolation.

3.1.3. Determination of growth curves

Determination of mycelial dry weight is a simple and accurate method for estimating fungal growth rate. Whole cultures from a flask at each time point were filtered by suction with predetermined weight Whatman 40 filter paper (W&R Balston Ltd, England) and mycelial pads remaining on the filter paper were oven-dried overnight at 54 °C, cooled in a desiccator and weighed (nearest 0.001 g). The results represented the mean value of three replicates.

3.1.4. Protoplast Isolation Procedures

Protoplasts were isolated by removing 0.25 g of mycelium (fresh weight), filtering this mass by suction, and washing the mycelium twice with sterile, distilled water and twice with 0.5 M mannitol or 0.5 M magnesium sulfate in 50 mM maleic-NaOH. Acetic acid-NaOH buffer (50 mM, pH 5.5) was also tried, but appeared to inhibit protoplast formation and was not used further.

The washed mycelium was resuspended in 3 ml of a 50 mM maleic-NaOH buffer solution which contained 0.4% Novozyme 234 (Industria A/S, Bagsaverd, Denmark) (a mixture of cell wall degrading enzymes) and 0.5 M mannitol or magnesium sulfate. Additional trials were performed using 0.3, 0.5, or 0.7 M magnesium sulfate as the osmotic stabilizer. The mixture was incubated at 30 °C for 2 h with occasional agitation. The effects of other variables were explored by altering the pH of the enzyme mixture

from 4.5-6.5 and the exposure time to the enzyme mixture from 60-180 minutes. Protoplast formation was initially observed by examining a small sample for evidence of protoplasts release. The presence of residual cell wall was monitored by reacting the suspension with fluorescein isothiocyanate coupled wheat germ agglutinin (FITC-WGA), a lectin specific for the n-acetylglucosamine residuals in chitin (Morrell et al., 1985). The cells were observed under a Leitz microscope equipped with filters specific for FITC. Cell walls fluoresced strongly, while protoplasts were only weakly fluorescent, indicating cell wall removal.

After enzyme treatment, 30 ml 50 mM maleic-NaOH buffer containing 0.5 M mannitol was added to dilute the enzyme, and the number of protoplasts were counted using a hemacytometer. Protoplast production was expressed as the number of protoplasts per gram mycelium fresh weight.

3.1.5. Protoplast Regeneration

The isolated protoplasts were diluted 1:1000 in a medium containing 0.5 M mannitol and 0-3.5% glucose in 50 mM maleic-NaOH buffer (pH 5.5). Cell wall regeneration was evaluated by incubating 2 ml of the protoplast suspension at 28 °C for 3-7 days, then spreading the solution on 2.0% agar containing 0.5% yeast extract, 0.5% malt extract, and 2.0% dextrose (MYG agar). The protoplasts were incubated for 10-15 days at 28 °C and observed for the presence of discrete fungal colonies, which served as the measure of successful regeneration.

Once protoplast regeneration was confirmed, the effects of colony age, glucose concentration, and pH on protoplast production and regeneration were examined for each of the test fungi.

3.1.6. Chemicals

Novozyme 234 was obtained from NOVO Industria A/S, Bagsaverd, Denmark; Malt and yeast extract from Difco (Detroit, MI); NaOH, $MgSO_4$, glucose and agar from Sigma Chemical Company (St. Louis, MO); FITC-WGA from Vector Laboratories Inc. (Burlingame, CA 94010). Maleic acid from Aldrich Chemical Co (Milluaukee, WI 53233); Mannitol from J.T.Baker Inc.(Phillipsburg, NJ 08865).

3.2. Results and Discussion

3.2.1. Protoplast Isolation

As shown in Fig 3.2., NOVOZYME 234 was effective for protoplast isolation for all four fungi tested, although protoplast yields differed among species. ß-1,3-glucanase and chitinase, the major active ingredients of this enzyme preparation, may play a major role in digesting cell walls of these basidiomycete fungi (Vries and Wessells,1973b).

Culture age strongly affected protoplast isolation (Fig 3.2). Yields of protoplasts declined substantially with increasing culture age. The optimal yields appeared to be

achieved with cultures in the early exponential growth phase for each of the four fungi (fig3.1.). These results agree with previous reports (Yanagi et al., 1985; Kitamoto et al., 1984; De Vries and Wessels, 1972). The significance of mycelial age for induction of protoplasts is most probably associated with changes in the hyphal walls. For example, the deposition of ß-1,3-glucan as an outer wall layer in older hyphae of A. nidulans may mask attack by lytic enzymes (Zonnevelad, 1972). Favorable protoplast yields were achieved when either $MgSO_4$ or mannitol were used as osmotic stabilizers, although the latter stabilizer appeared to be slightly less effective. Both stabilizers have been widely used for releasing basidiomycetes (Davis, 1975). The use of $MgSO_4$ as an osmotic stabilizer produced protoplasts of two types, large vacuolate types and small protoplasts lacking a large vacuole. This phenomenon was also noted by Wessels and De Vries (1972). Increasing the $MgSO_4$ concentration from 0.3 to 0.5 M resulted in increased protoplast yields with P.chrysosporium, G.trabeum and T.versicolor (Fig 3.1.). Yields decreased as the $MgSO_4$ concentration rose from 0.5 M to 0.7 M.

The 50 mM maleic acid-NaOH buffer (pH 5.5) was most suitable for protoplast release when stabilizer was present. Optimal protoplast release was achieved between pH 5.5-6.0 for all test fungi, although P. placenta was least affected by pH.

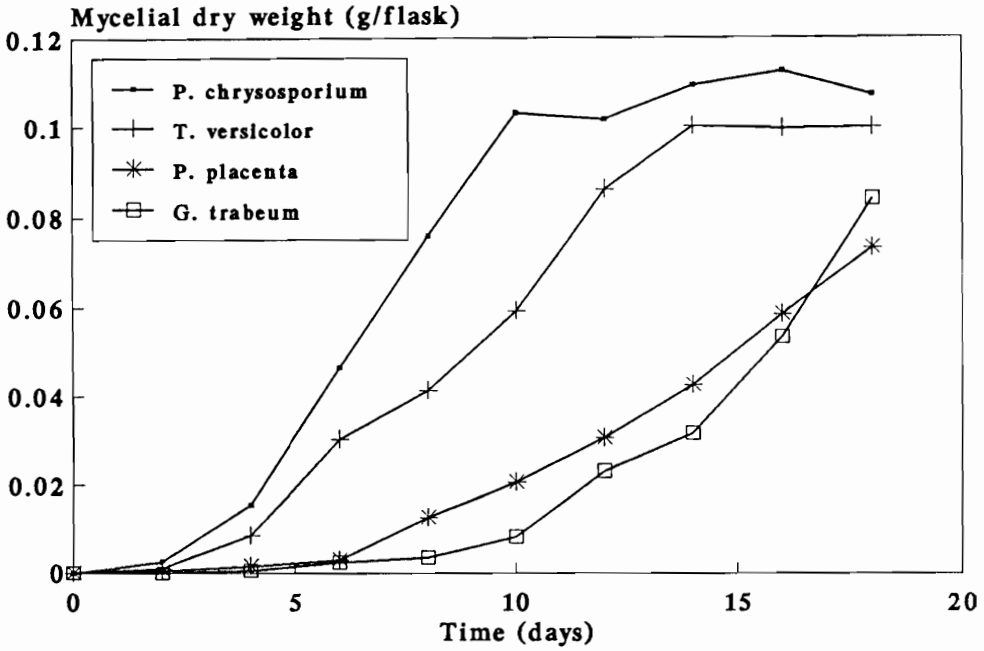

Fig 3.1. Mycelial dry weight production of 4 species of wood decay fungi grown on liquid medium containing 0.1% yeast extract, 1.0% glucose and 1% malt extract in stationary culture.

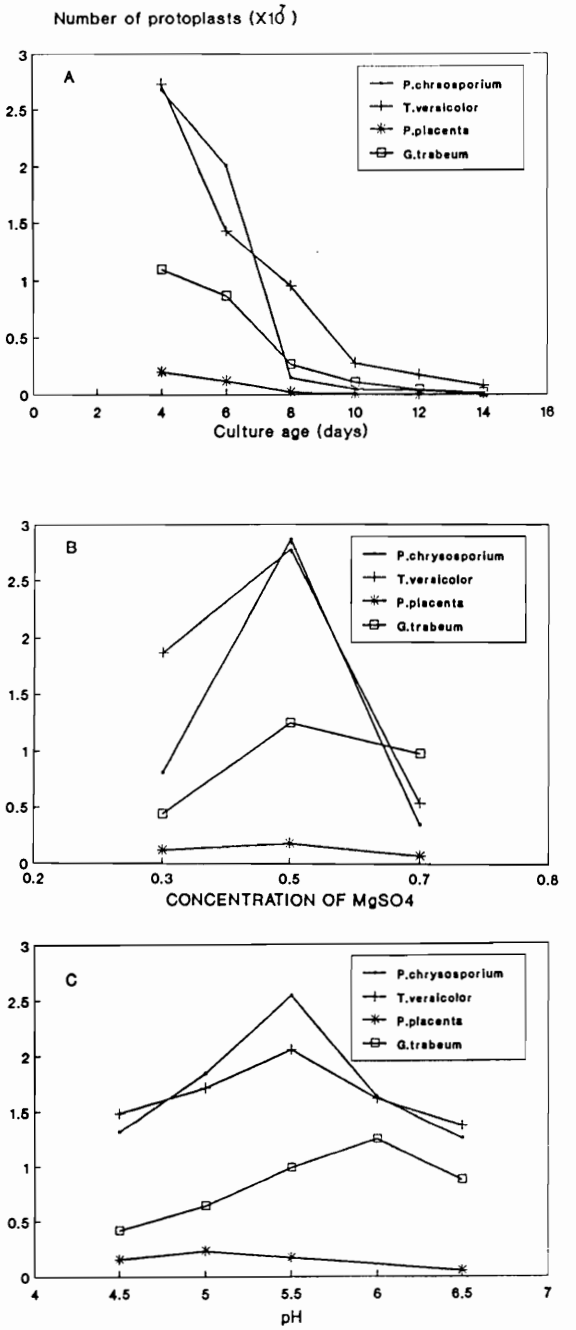

Fig.3.2. Effect of (A) hyphal age, (B) magnesium sulfate concentration in the osmotic stabilizer, and (C) pH of the medium on the production of fungal protoplasts from cultures of P.chrysosporium, T.versicolor, P.placenta and G.trabeum.

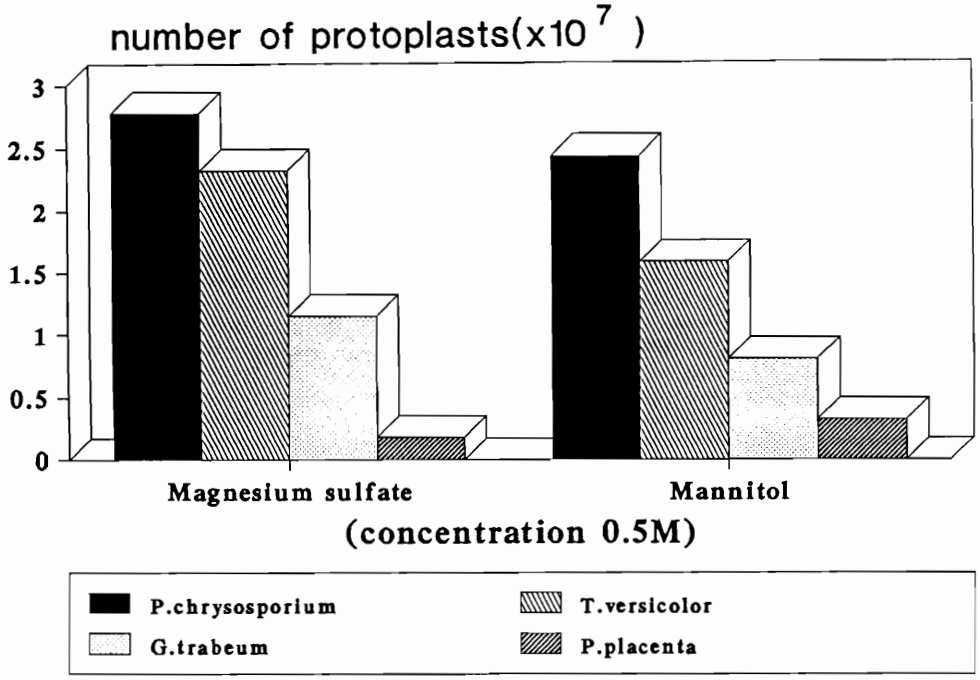

Fig 3.3. Effect of 0.5 M magnesium sulfate or 0.5 M mannitol as osmotic stabilizers for releasing fungal protoplasts from liquid cultures of 4 test fungi.

3.2.2. Protoplast Regeneration

Protoplasts isolated from all four fungi were able to regenerate cell walls when they were suspended in liquid medium or spread on agar MYG agar plates containing osmotic stabilizer. Hyphae originating from protoplasts developed into colonies within about a week. The protoplasts that failed to regenerate presumably either lacked nuclei or were damaged at some point during or after the enzyme treatment.

In contrast with protoplast isolation, regeneration improved with increasing culture age of the four species from which they were produced (fig 3.4.). The reason for this effect was unclear, since older cells would presumably be less plastic and therefore less capable of responding to the stress induced during protoplast formation.

Osmotic stabilizers also influenced protoplast regeneration. Magnesium sulfate appeared to inhibit regeneration, although it was effective for protoplast isolation, while 0.5 M mannitol was excellent for regeneration. As a result, mannitol was used as stabilizer in the remaining experiments. At the enzyme concentrations used, exposure to lytic enzymes for up to 3 h appeared to have little influence on protoplast regeneration of 3 of the test fungi, but G.trabeum was sensitive to exposure time (fig 3.4.C).

The choice of media pH should have substantial effect on regeneration, since media pH is directly related to activity of cell wall synthetases (Svoboda and Piedra, 1983). Increasing pH increased protoplast regeneration of G.trabeum and first improved, then decreased regeneration for remaining fungi (Fig 3.4.D). Plating efficiency (percentage

of cell regeneration) was highest at pH 6.0 for P.chrysosporium and at pH 5.0 to 5.5 for the other three fungi.

Glucose would be expected to be essential for regenerating protoplasts, since it is important in cell respiration and synthesis (Peberdy et.al., 1976; Vries and Wesseles, 1972). Glucose may also function as an osmotic stabilizer in medium. Increasing glucose concentration greatly enhanced protoplast regeneration, but the effect was slow and in some cases, protoplast regeneration declined at higher glucose concentrations. Gloeophyllum trabeum regeneration was highest in 1.5% glucose, while protoplast regeneration for the other three species was highest in 1.5-2.0% glucose (Fig 3.4.B). Regeneration was minimal in the absence of glucose. The difference in effects among species may reflect differential rates of glucose utilization.

3.3. Conclusion

Protoplasts can be produced from all of the wood degrading fungi tested and these protoplasts can regenerate into normal hyphae under suitable conditions. The availability of viable protoplasts provides an alternative system for studying fungicidal action. The uses of protoplasts in this aspect was assessed in further experiments.

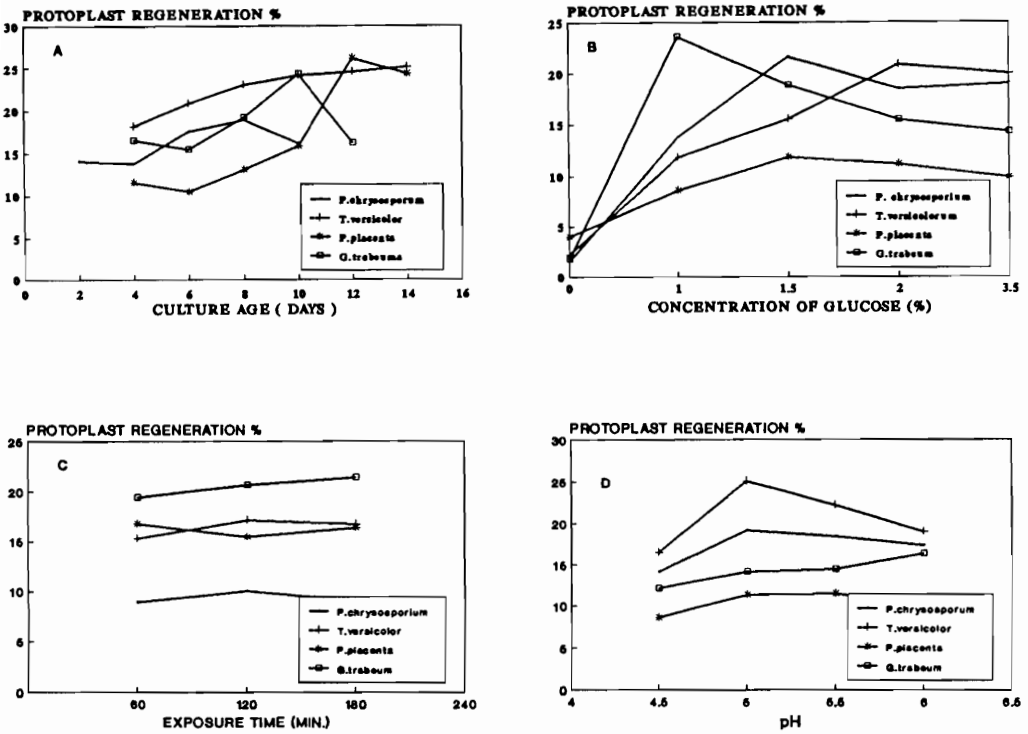

Fig 3.4. Effect of (A) hyphal age, (B) glucose concentration, (C) exposure time, and (D) pH of the extraction medium on the protoplast regeneration of 4 test fungi.

Section II

4. The Evaluation of the Use of Protoplasts for Studying Fungicidal Action

4.0. Introduction

Fungal protoplasts have been used for studying chemical sensitivity, uptake and transport phenomenon of toxicants; however, these applications have primarily been limited to yeast protoplasts. Only one report was involved with studies of Cu uptake using filamentous fungal protoplasts (Gadd, 1984).

The objective of this study was to characterize the effects of selected biocides on the physiologcal activity of fungal protoplasts and cell fragments.

4.1. Material and Methods

4.1.1. Organisms Tested and Preparation of Materials

Trametes versicolor and Postia placenta protoplasts were prepared according to methods described in Section 3.1.4 using 8 day old cultures of each fungus. Protoplast purification procedures were modified as follows: after enzymatic digestion for 2 h, the

crude protoplast preparation was filtered through 8 layers of cheesecloth to remove large mycelial debris. The filtrate was transferred into 50 ml centrifugation tubes, and centrifuged for 20 min at 2,500 xg. The supernatants was decanted and pelleted protoplasts were retained for bioassays.

Mycelial fragments were also used in this investigation for comparative purposes. The fragments were prepared by filtering 8 day old cultures through a 500 ml coarse scintered-glass filter and washing the hyphae with 50 mM malelic-NaOH buffer (pH 5.5). The mycelium was suspended in 200 ml of buffer, transferred into a Waring blender, and homogenized for about 4 seconds. The crude fragment preparation was filtered through 4 layers of cheesecloth and centrifuged for 20 min at 2,500Xg. The supernatant was discarded and the pellet was used for bioassays.

4.1.2. Fungicides Tested

Copper sulfate and azaconazole (1-[2-(2,4-dichlorophenyl)-1,3-dioxolan-2-methyl]-1H-1,2,4-triazole) were selected to evaluate fungicidal action on protoplasts and fragments. Copper is a non-specific biocide, and a major ingredient of chromated copper arsenate (CCA) and ammoniacidal copper zinc arsenate (ACZA). The fungicidal action of copper has been the subject of extensive studies (Ross, 1975; Gadd et al., 1987). Azaconazole, a triazole, has been recently introduced as a potential new wood preservative (Leclercq, 1983) and is up to 10 times as active as pentchlorophenol (Valcke and Messina, 1985). This chemical is generally recognized as a sterol inhibitor

(Sancholle et al., 1984), however, specific information on mechanisms of azaconazole against wood decay fungi is lacking.

Stock concentrations of copper sulfate 10, 25, 50, 75, 100. 125, 150 mM and 22, 55, 110, 165, 220, and 330 ug/ml of azaconazole were prepared. The solutions were sterile filtered through a 0.45 um diameter membrane (Millipore, Bedford, MA) and stored at 5 °C until needed. Final treatment concentrations were indicated in the results.

4.1.3. Effects of Fungicides on Viability of Protoplasts and Fragments and Mycelial Growth

Pelleted protoplasts were resuspended in 2 ml 50 mM maleic-NaOH buffer solution containing 0.5 M mannitol and 1 mM glucose to produce a suspension containing $2x10^7$ cells/ml. For comparative purposes, cell fragments were also suspended in 2 ml 50 mM maleic-NaOH solution containing 1 mM glucose without mannitol to give 3.7 mg cells (dry weight)/ml. At this concentration, fragments consumed O_2 at a rate similar to the protoplast suspension at a given time interval. The same concentrations of protoplasts and fragments were used in all bioassays, unless otherwise specifically stated. Copper sulfate and azaconazole, as concentrated stock solutions, were added to cell suspensions to produce required fungicide concentrations.After 2 hr incubation at 28 °C, the cell suspensions were diluted 1:250 in the same buffer solution. The viability of fungal protoplasts was assessed by adding 20ul of diluted protoplast or fragment solution in a 1.5 ml micro-centrifugation vial containing 980 ul of MYG liquid medium supplemented

with 0.5 M mannitol (for protoplasts) and poured over the surface of MYG agar plates. Colonies were counted after incubation for 10 days at 28 °C. The values relative to non-biocide exposed controls served as a measure of toxicity of fungicides on protoplasts. All procedures were performed aseptically and results represent the mean value of three replicates.

The effects of fungicides on mycelial growth were determined by placing small plugs (3mm diameter) cut from the actively growing edge of each culture into 25 ml of a MYG growth medium containing fungicides at required concentrations. The flasks were incubated for 9 days at 28°C days then mycelial dry weight was determined using procedures described in section 3.1.3.

4.1.4. Measurement of Oxygen Uptake

Oxygen uptake in the presence of glucose was measured with a YSI 5357 Clark-type polarographic oxygen probe 5357 with a model 5300 Oxygen Monitor (YSI, Ohio). The electrode chamber contained 1.8 ml of protoplast suspension ($2x10^7$ cells/ml or 3.7 mg of fragments (dry weight)/ml). The solution contained about 0.80 uM of dissolved oxygen when fully aerated at 30 °C. Thirty six ul of 50 mM glucose was added to the electrode chamber with a microsyringe to produce 1 mM final glucose concentration and the course of oxygen comsumption was recorded in order to determine effect of fungicides upon oxygen uptake.

4.1.5. Measurement of Glucose Depletion

Glucose depletion by protoplasts and mycelial fragments was measured by placing 3.84 ml of cell suspension at the required concentration into 10 ml test tubes containing 80 ul of 55 mM glucose (final concentration of glucose was 200 ug/ml) and 80 ul of fungicide stock solution at varying concentrations. One half ml of solution was withdrawn after stationary incubation for 15, 30 or 72 h at 28 °C,and centrifuged for 8.0 min. at 10,000 rpm using an Eppendorf centrifuge 5415 (Brinkmann instruments Co. CA). Supernatants were used for the determination of glucose by the o-Toluidine procedures according to glucose assay kit specifications (Hyvarinen and Nikkila, 1962).

4.1.6. Measurement of Copper Uptake and Potassium Leakage

Protoplasts and cell fragments were suspended in the 50 mM maleic-NaOH buffer solution (pH 5.5) containing 1 mM glucose to produce previously described cell concentrations. Copper sulfate and Azaconazole stock solutions were added to the cell suspension solutions and three replicates of each mixture were incubated for 2 h at 28 °C. Then, 1.5 ml of cell suspension was removed and centrifuged at 10,000 rpm for 5 min and the supernatant was used for the determination of copper on a Perkin Elmer model 4000 atomic absorption spectrometer operated at a wave length of 766.5 nm and using an air-acetylene flame. The pellets from each treatment sample were extracted in 1 ml of 6 N HNO_3 at 95 °C for 30 min. The solution was centrifuged for 5 min at

10,000 rpm and 0.8 ml of supernatant was diluted twice with 0.8 ml distilled water and 0.8 ml 3 N HNO_3. The diluted solution was analyzed for potassium using AA at a wave length of 324.8 nm and using an air-acetylene flame.Control samples without fragments or protoplasts were included in each experiment for comparative purposes.

4.1.7. Chemicals

Copper sulfate was obtained from J.T.Baker Inc.(Phillipsburg, NJ); Glucose assay kits, copper and potassium standard solutions for AA were obtained from Sigma Chemical Co. Ltd., (St. Louis, MO), and azaconazole was obtained from Jonssen Pharmaceutica,(Washington Crossing, NJ.

4.2. Results and Discussion

4.2.1. Effect of Copper Sulfate and Azaconazole on Mycelial Growth and Cell Viability.

Cu and azaconazole, at 2000 um and 4.4 ug/ml respectively, caused almost complete cessation of mycelial growth of both test fungi, while at 200 um and .22 ug/ml, respectively, there was little effect on growth (Fig 4.1. A and B). Higher concentrations of copper sulfate and azaconazole were required to kill T.versicolor and P.placenta cells (fig 4.1.C and D). Colonization of protoplasts and cell fragments of

both fungi were virtually inhibited within 2 h at 4000 uM $CuSO_4$ or 8.8 ug/ml azaconazole, respectively.

Protoplasts and cell fragments differed little in their sensitivity to fungicides (Fig 4.1.C and D) under the test conditions employed. For example, losses of cell viability were 62.9% and 67.4% for protoplasts and cell fragments of T.versicolor, or 65.4% and 70.1% for P. placenta protoplasts and fragments in the presence of 1000 uM copper sulfate, respectively. A dosage of 2.2 ug/ml of anaconazole resulted in viability losses of 47.6% and 57.1% for T. versicolor protoplasts and cell fragments, respectively. Loss of viability at the same dosage for P. placenta protoplasts and cell fragments was 49.1% and 55.9%, respectively. The similar toxicity between protoplasts and frgments is perplexing, since rigid cell walls may play an important role in biocide resistance by serving as either non-toxic binding sites or preventing direct entry of toxicants to cells (Gadd et al., 1984;). The loss of vulnerable, senescing cells during protoplast isolation might result in an increase of resistance of protoplasts to toxicants despite the absence of cell wall (Gadd et al., 1985). Furthermore, biocide absorption by a large number of non-viable protoplasts might reduce the effective fungicidal concentration, making protoplasts appear more resistant to fungicides.

Postia placenta has excellent resistance to copper (Zabel, 1953; Cowling, 1957); however, no striking differences in viability following copper sulfate exposure were noted between P.placenta and T.versicolor, a more copper sensitive fungus (fig 4.1. A, C). Levi (1968) suggested that the copper-resistance of P.placenta was due to the presence of sulphide and oxalic acid which precipitated copper as copper sulphide or

copper oxalate. It is probable that production of these compounds in the disturbed environment employed in the test was minimal, sharply reducing precipitation and detoxification.

4.2.2. Effects of Fungicides on Glucose Uptake of Cells

Both protoplasts and cell fragments were able to utilize glucose at the fungicide levels tested, but they showed different utilization patterns. Protoplasts initially utilized glucose more rapidly than cell fragments, glucose consumption by protoplasts declined much faster near the conclusion of the test. At 1500 uM copper sulfate or 4.4 ug/ml azaconazole, 20 ug/ml or 29 ug/ml of glucose, respectively, was depleted by T. versicolor protoplasts within 15 hr. Corresponding values were 5 and 14 ug/ml for T.versicolor cell fragments after exposed to the same chemical dosage. Glucose consumption at the same dosage was 31 and 37 ug/ml after 72 hr by T.versicolor protoplasts, and 79 and 112 ug/ml by T.versicolor cell fragments, respectively. The more rapid decline in glucose consumption may be due to higher sensitivity of protoplasts to fungicides, which might result in more rapid cell inactivation.

Copper sulfate had much higher inhibitory effects on glucose uptake and utilization than azaconazole, although the latter exhibited great effects on cell viability and biomass production (Fig 4.2 A). Losses of viability of T.versicolor protoplasts at 200 uM Cu and 0.44 ug/ml azaconazole were 4.2% and 24.3%, respectively, while glucose consumption was 57 ug/ml and 93 ug/ml within 30 h. This result suggests that copper

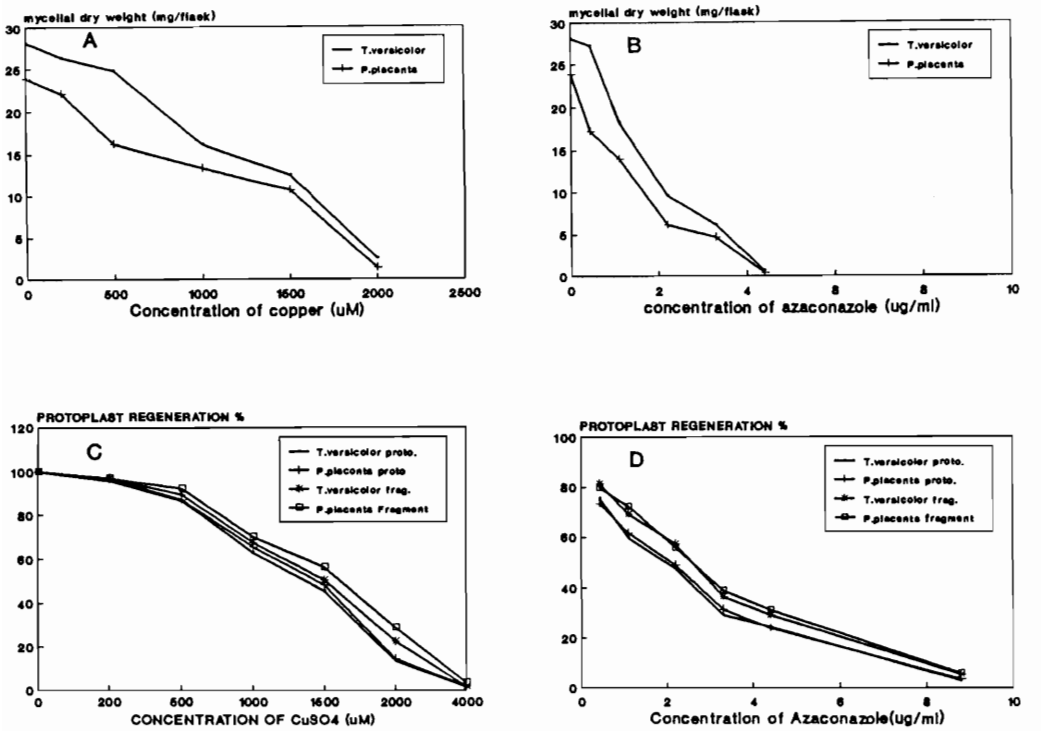

Fig 4.1. Effect of copper sulfate (A, C) or azaconazole (B, D) on biomass production (A, B) or cell regeneration (C, D) of T.versicolor and P.placenta following 2 hr exposure to chemicals. For plating efficiency of protoplast and cell fragments, values are expressed relative to controls. Plating efficiencies of control samples for T.versicolor and P.placenta protoplasts were 21.3% and 10.8%, and corresponding values for T.versicolor and P.placenta fragments were 90.5% and 87.4% , respectively.

might affect membrane function, indirectly limiting glucose uptake, or may directly affect glucose metabolism. Previous studies (Adam and Powell, 1957; Khovrychev, 1973) showed that Cu is a non-specific fungicide which disturbs membrane function by binding negatively charged enzymes on membranes. The potential effects of copper on glucose consumption become readily apparent when one considers the number of membrane associated steps in glycolysis and the Krebs cycle. Azaconazole, conversely, has a more subtle effect on cell physiology, and its initial effects on glucose consumption are much reduced.

4.2.3. Effect of Fungicides on Respiration

Oxygen uptake did not initially appear to be inhibited by addition of low levels of either fungicide, but the oxygen uptake was immediately inhibited in the presence of 10 mM copper sulfate or 13.4 ug/ml Azaconazole.

It is interesting to note that addition of 4 mM Cu initially stimulated the oxygen uptake of fragments of T.versicolor since higher copper sulfate concentrations inhibited oxygen uptake. Similar effects were noted by Hugo (1967) and Lambert and Smith (1976). Glucose uptake is a membrane mediated process. Increased respiration could be due to an increased glucose uptake as a result of membrane damage which facilitates substrate uptake without impairing enzyme activity (Lambert et al., 1976), however, this was not observed with protoplasts of T.versicolor.

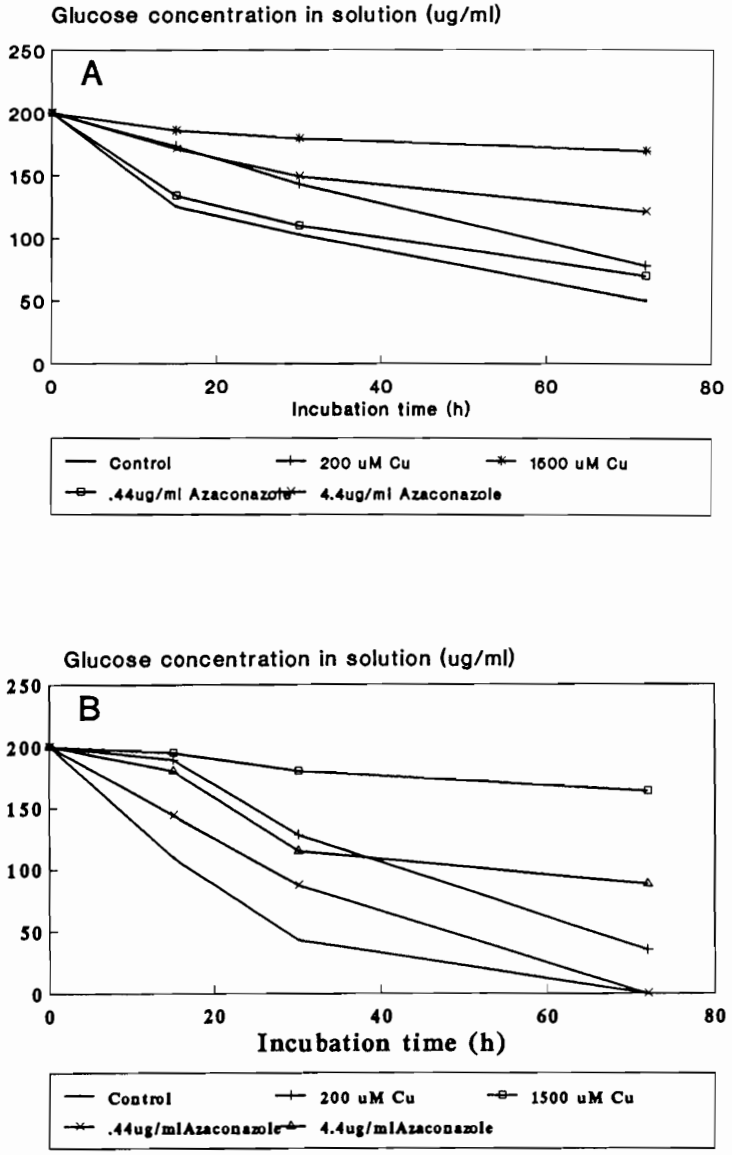

Fig 4.2. Effect of copper sulfate or azaconazole on glucose consumption of T.versicolor protoplasts (A) or cell fragments (B).

Table 4.1. Effect of copper sulfate and azaconazole at varying concentrations on oxygen uptake of T.versicolor protoplasts and cell fragments in the presence of 1 mM glucose.

Fungicide	Concentration	Time (min)[1]		O_2 satuation%
		Protoplast	Fragment	
$CuSO_4$	150 uM	31.2	30.6	0
$CuSO_4$	1500 uM	34.8	29.5	0
$CuSO_4$	4000 uM	44.6	26.3	0
$CuSO_4$	10,000 uM	71.0	51.4	64
azaconazole	.44 ug/ml	30.8	30.7	0
azaconazole	4.4 ug/ml	30.2	31.4	0
azaconazole	13.4 ug/ml	63.0	61.0	64
control		30.5	30.1	0

[1] Time refers to that required to decrease 100% oxygen satuation to corresponding satuation.

4.2.4. Effect on The Leakage of Potassium

Potassium release from cells has been used to monitor the effect of various biocides on membrane function of bacteria and fungi (Lambert and Hammond, 1973; Gale, 1974; Cope, 1980). Generally, membrane-active agents can disorganize or disturb membrane function by disassociating conjugated membrane proteins (Hugo,1967), resulting in the loss of small molecules, ultraviolet absorbing materials and ions such as amino acids, nucleotides and potassium ions from the cell.

Both fungicides induced potassium leakage, but the activity of azaconazole was much less pronounced (Table 4.1). Potassium levels for T.versicolor in protoplasts and cell fragments were 58.8% and 88.5%, respectively, of control samples, and 66.3% and 86.5%, respectively, for P.placenta protoplasts and cell fragments following a 2 hr exposure to 1000 uM $CuSO_4$. Higher levels of Cu were required to invoke the same amount of potassium leakage for P.placenta.

Increased cell leakage can have profound effects on membrane function and can also affect glucose metabolism in Escherichia coli (Miller et al., 1963). Thus, potassium levels may provide a broad guide to cell health and function.

Table 4.2. Effect of copper sulfate and Azaconazole on potassium leakage of protoplasts and fragments of T.versicolor and P.placenta following 2 hr exposure at 50 mM maleic-NaoH buffer of pH 5.5 and 28 °C.

Fungicide	Concentration	Potassium remaining level[2]			
		T.versicolor		P.placenta	
		Protoplasts	Fragments	Protoplasts	Fragments
$CuSO_4$	200 uM	.873	.913	.890	0.1009
$CuSO_4$	1000 uM	.544	.613	.801	.839
$CuSO_4$	1500 uM	.602	.662	.635	.551
azaconazole	.44 ug/ml	.877	.776	.920	1.029
azaconazole	1.1 ug/ml	.867	.776	.856	1.009
azaconazole	2.2 ug/ml	.901	.841	.886	1.003
azaconazole	3.3 ug/ml	.897	.820	.901	.999
azaconazole	4.4 ug/ml	.941	.79	.944	.979
control (initial)		1.105	1.103	1.100	1.050
control (final)		.925	.915	.905	.970

[2] Units for protoplasts and cell fragments are $2x10^7$ cells/ml and 3.7 mg dry weight/ml, respectively. At this level, cell fragments per ml gave an equvalent oxygen uptake protoplasts per ml.

4.2.5. Copper Uptake by Protoplasts and Fragments

Measurement of copper uptake by protoplasts and cell fragments of T.versicolor and P.placenta showed little difference in uptake under the concentrations tested (Fig 4.4.). Gadd et al.(1987) noted that copper uptake by Penicillium ochro-chloron deviated from a saturated curve due to protoplast lysis at higher copper concentrations. The results in the present study suggest that a significant amount of copper was absorbed by the protoplasts. For example, Cu uptake by T.versicolor and P.placenta protoplasts exposed to 1,000 uM $CuSO_4$ were 2.04 and 1.66 ug/10^7 cells, respectively. Previous studies suggest that there are two stages of copper uptake (Townsley and Ross, 1985). Process one represented physical adsorption of copper to negatively charged groups on the fungal surface, while phase two involved a slowly established energy dependent process of transport into the cytoplasm. Separation of cytoplasmic and membrane bound copper levels was not attempted in the current study since the intent of this trial was to compare the effects of copper sulfate on cell fragments and protoplasts.

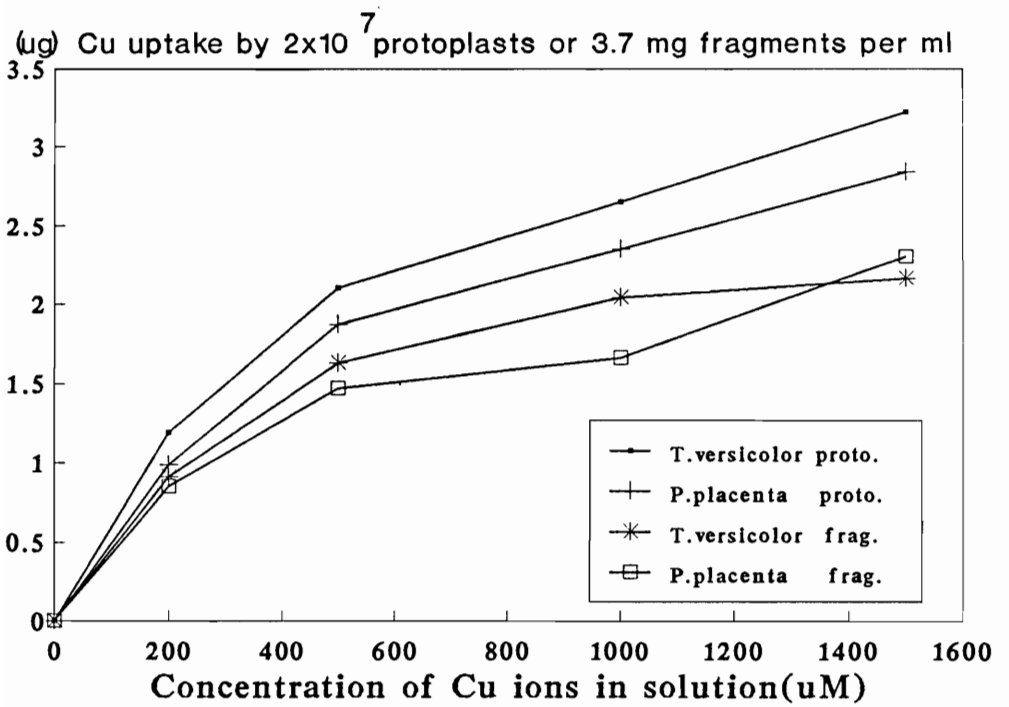

Fig 4.3. Copper uptake by protoplasts and cell fragments of T.versicolor or P.placenta following a 2 hr exposure to selected levels of copper sulfate in maleic-NaOH buffer at pH 5.5 and an incubation temperature of 28 °C.

4.3. Conclusions

The present studies indicate that isolated protoplasts can be used for studying physiology of fungi under chemical stress. Like cell fragments, freshly preparaed protoplasts can effectively utilize glucose and comsume oxygen. These properties of protoplasts along with their ability to regenerate provide a basis for studying the physiological action of fungicides.

The application of protoplast systems for studying the effects of fungicides on fungi produced results comparable with those obtained by using hyphal fragments in a series of bioaasays of fungicides. Generally, similar effects of fungicides on protoplasts and cell fragments were observed, although protoplasts showed a relative higher sensitivity in most cases. However, fungal protoplasts were much less sensitive than plant protoplasts (Breiman and Galun, 1981). The loss of vulnerable and senescing cells, alteration of some properties of protoplasts during or after lytic enzyme digestion or biocide absorption by non-viable protoplasts might result in reducted sensitivity of protoplasts to chemicals.

5. General Evaluation

Previous studies suggest that protoplasts have excellent potential for use in studies of fungicidal action, where the role of cell wall, permeability, transport mechanisms, and homogeneity of system associated with filamentous fungi may be major considerations. However, this study suggested that results obtained with protoplasts in place of mycelium should be interpreted with caution and further comparative studies are required. In addition, further studies are needed to more fully delineate the optimaum condition for physiological activity of protoplasts of various wood degrading fungi. As these conditons are identified, the application of protoplasts for studying the processes of wood degradation and effects of biocides on cell health will become more practical.

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