FULL PAPER

N. Roth-Bejerano · S. Mendlinger · V. Kagan-Zur

Effect of calcium on growth of submerged *Terfezia boudieri* mycelium

Abstract The effect of Ca^{2+} on mycelial growth in *Terfezia boudieri* was studied. *Terfezia boudieri* Chatin (Ascomycotinae) occurs in mycorrhizal association with *Helianthemum* shrubs in deserts with calcareous soils. External Ca^{2+} stimulated mycelial growth in both liquid media and solidified substrates. The response to Ca^{2+} was very faint in well-aerated culture but pronounced in mycelia immersed in the medium, indicating dependence on mycelial aeration. 2,4-Dinitrophenol (DNP), an inhibitor of oxidative phosphorylation, and succinate, a potential cytoplasm acidifier, inhibited mycelial growth but enhanced the stimulatory effect of Ca^{2+}. This effect was reduced by the Ca^{2+} channel blocker verapamil.

Key words Aerobic conditions · Hypoxia · Verapamil

Introduction

The fungus *Terfezia boudieri* Chatin inhabits the arid regions of Morocco (Lahsen Khabar, personal communication), Saudi Arabia (Bokhary and Parvez 1992), Iraq (Abdullah et al. 1989), and Israel (Holdengraeber et al. 2001), all regions characterized by high-pH calcareous soils. External Ca^{2+} has been shown to stimulate hyphal elongation in certain fungi (Jackson and Heath 1993; Dicker and Turian 1990). In *Saprolegnia ferax* (Hyde and Heath 1997; Yuan and Heath 1991), *Achlya* spp. (Reiss and Herth 1991), and *Neurospora crassa* (Dicker and Turian 1990; Schmid and Harold 1988), this stimulatory effect was found to be conditional on the presence of a high tip-to-base gradient of

N. Roth-Bejerano (✉)
Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel
Fax +972-8-6472992
e-mail:nuritrb@bgumail.bgu.ac.il

S. Mendlinger · V. Kagan-Zur
The Institutes for Applied Research, Ben-Gurion University of the Negev, Beer Sheva, Israel

Ca^{2+}. It was also found that verapamil, a voltage-dependent Ca^{2+} channel blocker (White 1998), inhibited the elongation of hyphae in both *N. crassa* and *S. ferax* (Dicker and Turian 1993; Jackson and Heath 1993, respectively). Robson et al. (1996) reported that an alkaline pH near the growing tips is essential for hyphal elongation of *N. crassa,* except at high Ca^{2+} concentrations. These findings are in line with those of Bachewich and Heath (1997), who showed that acetic acid, which slightly lowered the cytosolic pH near the hyphal tips of *S. ferax*, decreased the hyphal growth rate. In higher plants, lowering of the cytoplasmic pH and ATP reduction have been shown to be caused by hypoxia (Ratcliffe 1997), thus potentially linking low pH and low growth rate to oxygen shortage. Subbaiah et al. (1994) reported that anoxia reduced the vitality of suspension-cultured maize cells and stimulated Ca^{2+} release from internal pools to the cytosol. Blockers of Ca^{2+} release further reduced vitality, which could, in turn, be restored by external Ca^{2+}. It seems, therefore, that Ca^{2+} is essential for cell survival under conditions of oxygen restriction.

We observed that growth of *T. boudieri* mycelium decreased in liquid media but could be restored by the addition of Ca^{2+}. We surmised, therefore, that this phenomenon may be related to a decrease in oxygen availability to the immersed mycelium. Thus, in the study reported here we examined the effect of Ca^{2+} on mycelial growth of *T. boudieri* under a range of conditions suspected of modifying Ca^{2+} uptake, e.g., under both aerobic conditions (uncovered mycelia placed in solid or liquid medium) and hypoxia (mycelia fully immersed in solid or liquid media). In view of the findings regarding the effects of O_2 deprivation in plant tissue (reduced cytoplasmic ATP, lowered cytoplasmic pH), we lowered the ATP level with 2,4-dinitrophenol (DNP) and acidified the cytoplasm with succinate, and then examined the effect of each of these measures on mycelial growth and on Ca^{2+} demand under aerobic conditions on solid media. The effect of verapamil was studied under hypoxic conditions.

Materials and methods

Fungal material

The material used in these experiments consisted of *T. boudieri* mycelium initially isolated from gleba of fresh fruit bodies onto Fontana medium (Bonfante and Fontana 1973). The stock was grown in Petri dishes containing 25 ml of this medium at 25°C. The experiments were started using 1-cm^2 plugs from 4- to 6-week-old colonies.

The mycelia of *T. boudieri* grow very slowly, at a rate of 0.6 mm/24 h, versus 12 mm/24 h for *N. crassa* as calculated from data of Levina et al. (1995). Our experiments were therefore run over a period of about 1 month, a relatively long time compared with similar experiments reported in the literature (Bachewitch and Heath 1997).

Growth media and conditions

Fontana medium modified with 2 mM Tris-MES (2-(N-Morpholino)ethanesulfonic acid) buffer, pH 6.5, was used. The final pH after autoclaving was 6.0. In the liquid media experiments, either 10 or 30 ml Fontana medium was placed in 50-ml Erlenmeyer flasks without or with addition of 1 or 5 mM CaCl$_2$. Plugs placed in the 10-ml volume were exposed to air while those placed in 30 ml were fully immersed in the medium, providing different degrees of aeration for the growing mycelium. The experiment was conducted at five different times with each time period constituting a temporal block. Each block consisted of 4–6 replicates per treatment. The results were analyzed as a three-way mixed ANOVA with blocks as a random factor. In the experiments on solidified media, aeration was modulated either by placing the initial plugs on Fontana medium solidified with 1.5% agar (nonsubmerged culture), or by placing them on Fontana solidified with 0.65% agar and subsequently covering them with a 5- to 7-mm-thick layer of the same medium at a temperature of 30°–35°C (immersed culture). Immersing the mycelium had the effect of reducing oxygen concentration from 300 mg/l in air to 8.5 mg/l (Lide and Frederikse 1997).

For statistical analysis, the positioning of the plug and the addition of calcium constituted two fixed factors; the time block constituted a random factor. The experiment was conducted three times, each repeat representing a block with five replicates per treatment.

Inhibitor experiments

The effects of DNP, succinate, and verapamil were studied in solidified medium. Our decision to use succinate, which is a product of regular aerobic metabolism, was dictated by the length of the experiment and our desire to avoid any harmful effects on growth. Although, as mentioned earlier, at low pH weak organic acids acidify mycelial cytoplasm (Bachewitch and Heath 1997), we chose to use succinate at pH 6.0 based on findings of Fonteriz et al. (1991) that pro-

pionic acid is capable of lowering cytoplasmic pH even at pH values higher than its pK (pH 7.4). The DNP, succinate, and verapamil were dissolved in the medium. DNP and verapamil were sterilized through a 0.45-μm filter. The DNP experiment was conducted in three levels of DNP (0, 0.1, and 0.25 mM) and three levels of added CaCl$_2$ (0, 1, and 5 mM) in submerged mycelia. Each treatment was replicated five times. The data were analyzed as a one-way ANOVA. The succinate experiment was performed in submerged plugs using four treatments: two levels of succinate (0 and 2.5 mM) and two levels of CaCl$_2$ (0 and 1 mM). The experiment was conducted in two different time blocks with five replicates per block. The data were analyzed as a three-way mixed-model ANOVA.

The inhibitory effect of verapamil was studied in mycelia immersed in standard and diluted Fontana medium (95 and 15 mosmol/kg, respectively). The effect of verapamil was estimated by comparing mycelial growth without and with verapamil for three or two levels of added Ca^{2+}. Percent inhibition by verapamil was calculated by comparing growth of verapamil-treated with that of nontreated plugs, taking the size of the controls as 100%. The results were analyzed as a linear regression.

Estimation of growth

Because *T. boudieri* grows very slowly, the first estimate of growth was made after 3–4 weeks. In the case of liquid media, the mycelium was separated from the agar plug and weighed after excess solution had been removed by gentle pressing between two sheets of tissue paper. For solidified media, colony radius was measured.

Results

Effect of Ca^{2+} in submerged and aerated cultures

Addition of 1.0 mM of external Ca^{2+} enhanced growth of mycelium immersed in liquid medium. When we compared mycelial growth in the presence of additional Ca^{2+} in submerged and nonsubmerged culture, we found that growth was significantly affected by both factors examined, namely added calcium and aeration. All main treatments and primary interactions were significant; secondary interaction was insignificant. In the absence of added Ca^{2+} mycelial growth was more rapid in the smaller volume of medium (plug exposed to air; Fig. 1A). The addition of Ca^{2+} promoted growth in the 10-ml (nonimmersed) treatment slightly, although not significantly, whereas in the 30-ml treatment (immersed) it resulted in a significantly higher growth rate. On nonsupplemented solidified medium, the exposed mycelial plugs showed significantly faster growth than the immersed plugs (Fig. 1B). Although the addition of Ca^{2+} caused a small but insignificant increase in growth in nonimmersed plugs, its stimulatory effect in immersed plugs was significant.
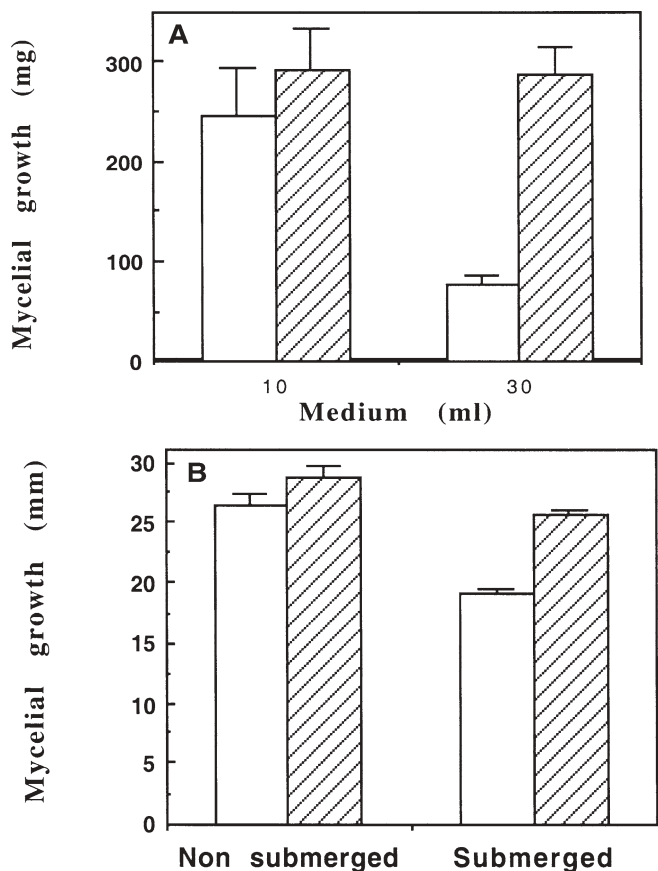
Fig. 1. Effect of Ca^{2+} on mycelial growth in immersed and aerated cultures. **A** Mycelium not immersed in liquid medium (10 ml) or immersed in liquid medium (30 ml) in a 50-ml Erlenmeyer flask. **B** Mycelium placed on or immersed in solid agar. *Clear bars*, without additional Ca^{2+}; *hatched bars*, 1 mM Ca^{2+}

Effect of DNP and succinate on mycelial growth and response to Ca^{2+}

In the DNP–calcium experiment, all main treatments and interactions were significant. DNP inhibited mycelial growth, and addition of Ca^{2+} decreased or suppressed this inhibition (Fig. 2A). Succinate inhibited mycelial growth and increased its sensitivity to external Ca^{2+} (Fig. 2B).

Effect of verapamil, a Ca^{2+} channel blocker

Verapamil inhibited mycelial growth, although percent inhibition decreased with time. Supplementation with 1 mM Ca^{2+} had only an insignificant effect on inhibition by verapamil, whereas 5 mM Ca^{2+} reduced it significantly at an early stage of the experiment (Fig. 3). Medium osmolarity mediated mycelial growth response to verapamil. Percent inhibition in low osmoticum was smaller than in high osmoticum (Fig. 4).

Verapamil not only inhibited mycelial growth, it also affected hyphal morphology, inducing multiple tip branching. Ca^{2+} abolished the inhibitory effect of verapamil on hyphal growth and branching (data not shown).
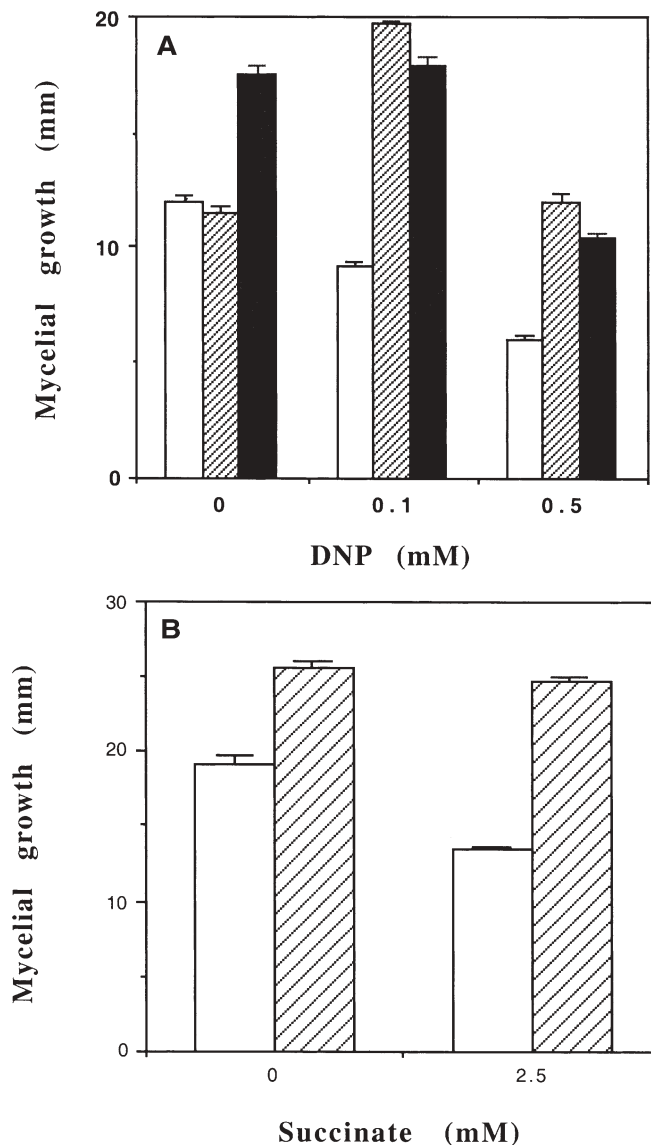

Fig. 2. Effect of Ca^{2+} on mycelial growth in plugs immersed in solid medium (**A**) modified with 2,4-dinitrophenol (DNP) and (**B**) modified with succinate. *Clear bars*, without additional Ca^{2+}; *hatched bars*, 1 mM Ca^{2+}; *solid bars*, 5 mM Ca^{2+}

Discussion

It is known that Ca^{2+} is essential for hyphal tip growth but, as mentioned by Jackson and Heath (1993), its exact function is poorly understood. It has been suggested that Ca^{2+} affects growth by binding with different proteins and thereby influencing metabolism, or else by regulating the F-actin network (Jackson and Heath 1993). In *T. boudieri*, the amount of Ca^{2+} contained in Fontana medium (0.17 mM) seems to be adequate for cultures on solid media or in liquid culture where the mycelia are not immersed in the liquid. Under such conditions, further elevation of Ca^{2+} level has little effect on mycelial growth (Fig. 1A,B). In immersed mycelia, on the other hand, Ca^{2+} supplementation stimulates mycelial growth. This effect may be related to the
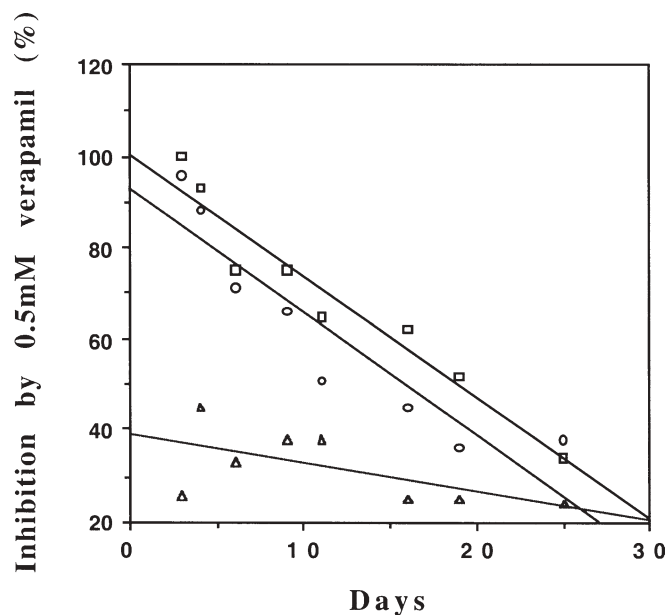
Fig. 3. Inhibition of mycelial growth by verapamil in plugs immersed in solid media and reversal of verapamil-induced inhibition by external Ca^{2+}. *Squares*, without additional Ca^{2+}; *circles*, 1 mM Ca^{2+}; *triangles*, 5 mM Ca^{2+}. Percent inhibition by verapamil was calculated by comparing growth of verapamil-treated plugs with that of control plugs, taken as 100%
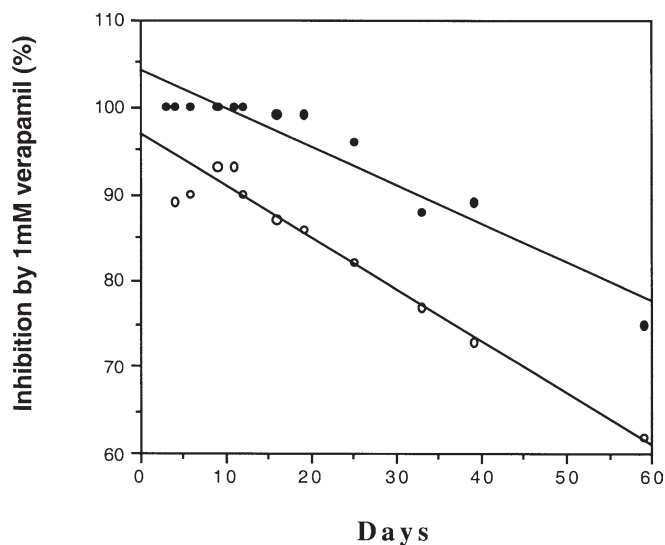

Fig. 4. Inhibition of mycelial growth by verapamil in plugs immersed in solid Fontana: full versus diluted medium. *Open circles*, Fontana medium (95 mosmol/kg); *solid circles*, diluted Fontana (15 mosmol/kg). Percent inhibition calculated as in Fig. 3

hypoxic conditions resulting from the poor solubility of oxygen in the medium. Oxygen deprivation has been shown to decrease ATP level, lower cytoplasmic pH (Ratcliffe 1997), and elevate cytoplasmic Ca^{2+} (Subbaiah et al. 1994) in plant tissues. Although the sequence of events remains unclear (Ratcliffe 1997; Subbaiah et al. 1994), it appears likely that oxygen and these three parameters are linked in some way. Our finding that both DNP and succinate increased mycelial

demand for Ca^{2+} in nonimmersed cultures (Fig. 2A,B) suggests that a similar relationship prevails in *T. boudieri*. Robson et al. (1996) found that cytoplasmic acidification of growing tips inhibits elongation, and Bashewich and Heath (1997) showed that although acetic acid decreases internal pH by only a small amount, from 7.2 to 6.8, it inhibits hyphal growth. We venture to propose that the inhibition of elongation associated with immersion and the presence of DNP and succinate is linked to cytosolic acidification of the hyphae. It could be that external Ca^{2+} restores mycelial growth by elevating cytosolic pH (Roncal et al. 1993).

Subbaiah et al. (1994) report that the increase in cytosolic Ca^{2+} observed under hypoxia takes place at the expense of intracellular stores and hence is unaffected by external Ca^{2+} or verapamil. Contrary to these results, in *T. boudieri* the required Ca^{2+} is drawn from external stores, as supported by the finding that verapamil inhibited mycelial growth and altered hyphal morphology, and furthermore that elevation of external Ca^{2+} decreased or reversed these effects (Fig. 3). Recovery from verapamil inhibition depended on Ca^{2+} concentration, with only the high level, 5 mM, having a significant effect (Fig. 3). These results are in line with findings of Dicker and Turian (1990) for *N. crasssa* and of Hudecova et al. (1994) for *Botrytis cinerea*. The reduced inhibition by verapamil in diluted Fontana (Fig. 4) implies that low osmoticum enables Ca^{2+} penetration to the hyphae by pathways other than those inhibited by verapamil. A possibility that cannot be ruled out is that osmotic shock opens stretch-activated channels similar to those found in the aquatic oomycete *Saprolegnia* (Lew 1998).

The fact that hypoxia evokes Ca^{2+} requirements in a terrestrial fungus may be a consequence of its adaptation to its habitat. *T. boudieri* is a mycorrhizal fungus living in association with desert shrubs, partly inside the mycorrhized roots and partly in the soil. The extraradical mycelium expands mainly in the winter season preparatory to production of fruit bodies the following spring. Winter floods, a not infrequent phenomenon in the Israeli desert in rainy years, may create hypoxic conditions that could inhibit mycelial growth. In such a situation, stimulation of Ca^{2+} penetration would restore growth and enable the life cycle of the fungus to proceed to its culmination, the production of fruit bodies with ascospores.

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