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# The Role of Retinol in the Initiation of Sporangiophores of *Phycomyces blakesleeanus*

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**Abstract.** The initiation of sporangiophores of *Phycomyces* was analyzed under oxygen-limiting conditions. Mutants lacking β-carotene have a higher oxygen threshold than the wild type depending on the residual amount of β-carotene. The supersensitivity to low oxygen tension is specific for sporangiophore initiation and can be suppressed by addition of either retinal, retinol or retinol acetate to the medium. It is suggested that retinol is a natural regulator of differentiation in *Phycomyces*.

**Key words:** β-Carotene mutants – Oxygen requirement – *Phycomyces* – Retinol – Sporangiophore initiation.

## Introduction

In several fungi, blue light controls the initiation of spore bearing structures (Uebelmesser, 1954; Sargent and Briggs, 1967). Even the initiation of sporangiophores (spph) of *Phycomyces* is subjected to this type of blue light control (Bergman, 1972; Thornton, 1975). A partialaction spectrum and genetical evidence indicate that the same photoreceptor, being functional in the phototropism of *Phycomyces* – most likely a flavin – is even responsible for the light induced spph initiation (Bergmann, 1972; Bergman et al., 1973).

Recently we began to study spph initiation of *Phycomyces* in small glass houses in which the differentiation is autoinhibited by changes of the gas atmosphere caused by the metabolism of the mycelium (Russo, 1977). In this system too, blue light stimulates the initiation of spph, as the light negates the inhibition exerted by the gas. We found that β-carotene lacking mutants exhibited a higher sensitivity to the atmosphere in the glass house than the wild type. This finding induced us to further analyze the relationship between the capacity to synthesize β-carotene and spph initiation in the glass house system.

In this article we will show that under limited oxygen concentrations, differences in spph initiation between wild type and β-carotene lacking mutants become manifest. We suggest that retinol is a natural regulator of differentiation in *Phycomyces*.

## Material and Methods

### *Strains*

All strains used were derivatives of *Phycomyces blakesleeanus* NRRL1555 and are listed in Table 1.

### *The Closed System and Reconstitution Experiments*

The closed system consists of a 1l-glass beaker with straight walls; such a beaker is inverted on a petri dish which fits the beaker. When the inoculated shellvials are in the beaker, the remaining space between petri dish and glass beaker is filled with water to make it airtight. In order to avoid evaporation of water, several layers of parafilm are put over the margin of the petri dish. The number of spph is determined 120 h after inoculation.

In the reconstitution experiments, shellvials containing 50 hour-old mycelia are transferred to a 3l desiccator. Each desiccator has an inlet and an outlet such that the atmosphere can be rapidly changed with a given stream of gas. Different oxygen concentrations are achieved by filling the desiccator with nitrogen and later adding the required amount of oxygen with a syringe. Spph are counted 120 h after inoculation.

### *Measurement of Oxygen Concentrations*

Oxygen concentrations in the closed beaker were determined with a Gilson Oxygraph, Model IC-OXY, equipped with a clark electrode. Since this electrode measures only in liquid the following procedure was applied. With a 5 ml syringe 2.5 ml samples of gas were taken out from the atmosphere of the closed beakers; for this purpose the beakers had a little hole with a rubber stopper through which the needle of the syringe passed. The 2.5 ml gas

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**Table 1**

| Designation | Genotype | Phenotype | Reference |
|---|---|---|---|
| NRRL1555 | wild type (−) | yellow | Bergman et al., 1973 |
| C2 | *carA5* (−) | white, no phytoene | Meissner, Delbrück, 1968 |
| C148 | *carA5 madC119* (−) | white, phototropic, defective | Bergman et al., 1973 |
| C9 | *carR21* (−) | red, accumulation of lycopene | Meissner, Delbrück, 1968 |
| C5 | *carB10* (−) | white, accumulation of phytoene | Meissner, Delbrück, 1968 |
| C173 | *carB32 carR21* (−) | white | Ootaki et al., 1973 |
| C171 | *carA30 carR21* (−) | white | Ootaki et al., 1973 |
| C6 | *carA12 carR27 madF48* (−) | white, phototropic, detective | Bergman et al., 1973 |

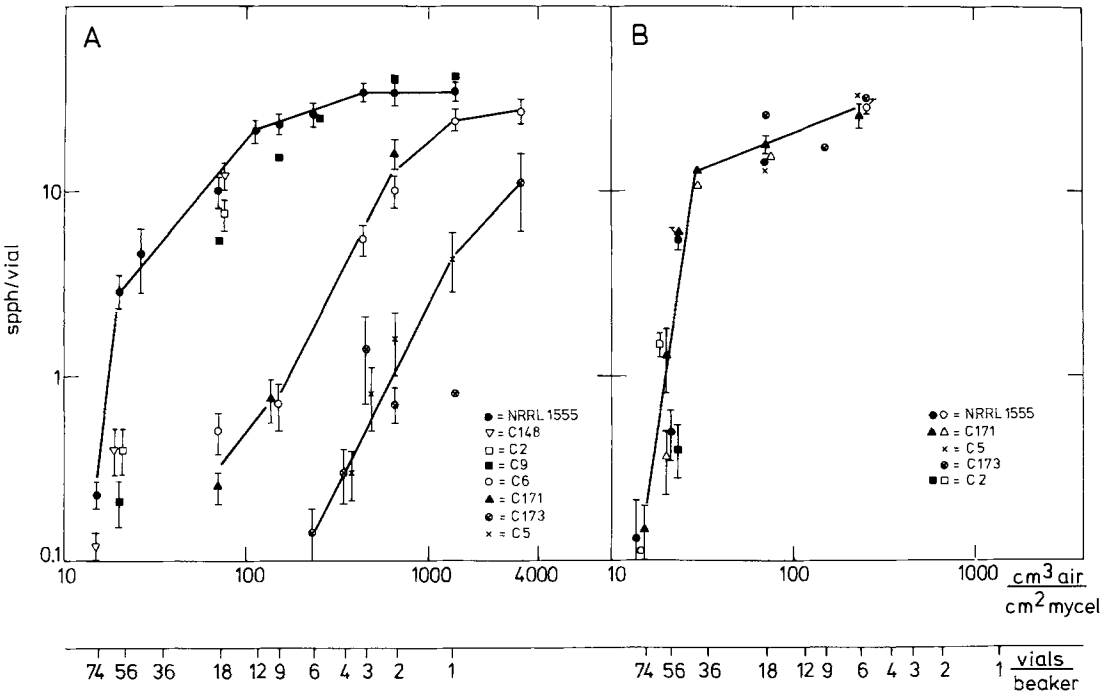


**Fig. 1 A and B.** Inhibition of spph formation in the closed system; behaviour of wild type and β-carotene lacking mutants. The standard error of the mean of at least 4 experiments is indicated by the bars. Points without bars represent the mean of two experiments. **A** Medium without retinol acetate, **B** closed symbols = addition of 250 μg $ml^{-1}$ retinol acetate; open symbols = addition of 250 μg $ml^{-1}$ retinol to the medium

samples were equilibrated inside the syringe with 2.5 ml water which had been saturated with nitrogen. 1.85 ml of this equilibrated water was added to the oxygen electrode chamber and the concentration of oxygen was determined.

*Chemicals*

Retinol acetate, retinol and retinal were purchased from Sigma (Chemie GmbH, München); for addition to the medium 300 mg of these compounds were dissolved in 5 ml of Tween 20 (Serva, Heidelberg) and 2.5 ml ethanol and 5 ml of this mixture was poured into 800 ml liquid agar to give a final concentration of 250 μ $gml^{-1}$. All these media were freshly prepared for immediate use.

*Media*

In all experiments *Phycomyces* was grown on potato dextrose agar; for details see Russo (1977).

## Results

### *Inhibition of spph Formation in the Closed System*

The formation of spph of *Phycomyces* can be completely inhibited in a closed system (Russo, 1977). The efficiency of this inhibition depends on the air volume available per unit area mycelium. Fig 1 A shows how the number of spph depends on the number of inoculated shellvials per beaker (volume = 1 liter). The shellvials were inoculated with 5–15 heat induced spores, put into the beaker and were continuously kept in the dark. After 70 to 90 h spph appeared which stay in stage I (0.5–1.5 cm, no sporangium) when the beaker contains 40–72 vials; with less than 40 vials per beaker, about 10% to 20% of the spph reach stage IV (spph with mature sporangium). It can be seen from Fig. 1 A that in the wild-type

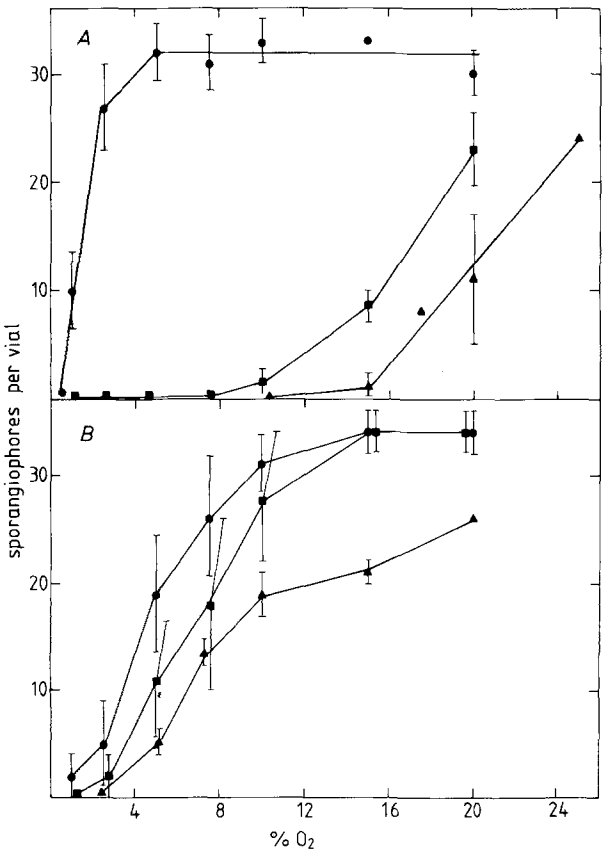


**Fig. 2A and B.** Oxygen threshold and effect of retinol acetate on spph formation; behaviour of wild type and β-carotene lacking mutants. The bars indicate the standard error of the mean of 4 experiments; points without bars represent the mean of two experiments. **A** Medium without retinol acetate, dark. **B** Medium containing 250 μg $ml^{-1}$ retinol acetate, dark. ●—● NRRL1555; ■—■ C171; ▲—▲ C173

NRRL 1555, little inhibition takes place with 1 to 40 vials per beaker; with more than 40 vials the number of spph rapidly decreases to about 0.2 spph per vial.

Mutants deficient in the synthesis of β-carotene can clearly be divided into three classes: mutants C2 (*carA5*) and C9 (*carR21*) are slightly more sensitive to the gaseous atmosphere in the beaker than the wt; mutants C171 (*carA30 carR21*) and C6 (*carA12 carR27 madF48*) are 5–10 times more sensitive than wt; C5 (*carB10*) and C173 (*carB32 carR21*) are 20 times more sensitive. The three groups of mutants differ in their β-carotene content: the former class contains 1.2% (C2) and 0.2% (C9) of β-carotene (Meissner and Delbrück, 1968); mutant C171 contains about 0.1% (Ootaki et al., 1973) and C5 and C173 less than 0.05% (Presti et al., 1977).

### *The Effect of Retinol in the Closed System*

The supersensitivity of C171 and the other mutants could either be due to lack of β-carotene itself or else to lack of a metabolite of it. Since β-carotene is very poorly taken up by the mycelium, we added retinol to the medium; retinol is postulated to be the degradation product of β-carotene (Olson and Hayaishi, 1965). Addition of either retinol acetate or retinol suppresses the supersensitivity of mutants C171, C173, and C5 (Fig. 1B).

**Table 2.** Effect of retinol and retinal on the oxygen threshold of strain C173 in the dark

| | Oxygen threshold |
|---|---|
| Control | 15% |
| 50 μg $ml^{-1}$ retinol | 7.5% |
| 125 μg $ml^{-1}$ retinol | 6% |
| 50 μg $ml^{-1}$ retinal | 2.5% |
| 125 μg $ml^{-1}$ retinal | 2.5% |

Conditions as described in Fig. 2. For each quantity of retinol and retinal a threshold curve was established analog to the one in Fig. 2

### *Oxygen Dependence of spph Formation and Retinol*

In reconstitution experiments we tested whether a lack of oxygen was involved in the inhibition of spph formation. Dark-grown mycelia on shellvials were transferred 50 h after seeding to a 3 l desiccator; each desiccator contained 2 vials with wt and 2 with C171 such that the volume of air available per vial was sufficient to avoid interference of any self-produced gas with the test gas (see Fig. 1). In the case of C173 only one vial per desiccator was given. 120 h after seeding, the number of spph per vial was determined. Fig. 2A shows the oxygen dependence of spph formation for wt NRRL1555, C171 (*carA30 carR21*) and C173 (*carB32 carR21*). The oxygen threshold for the wt NRRL1555 is 0.5%, 10% for C171 (*carA30 carR21*) and 15% for C173 (*carB32 carR21*).

Since in the beaker system the β-carotene mutants are phenotypically suppressed by addition of retinol acetate (Fig. 1B), we tested whether retinol acetate has any influence on the oxygen sensitivity. Figure 2B. shows the oxygen thresholds of NRRL1555, C171 and C173 after addition of 250 μg $ml^{-1}$ retinol acetate into the medium. It is evident that the mutants can be restored almost to the normal threshold of the wild type. As a control we showed that this effect was not an artefact of the solvent Tween 20 and ethanol. Table 2 shows the capacity of retinol and retinal to restore the oxygen threshold of C173: clearly retinal is more efficient than retinol.

Figure 3 shows the effect of retinal on the spph formation on various β-carotene lacking mutants, which differ in their residual β-carotene synthesis. It is evident that with decreasing β-carotene concentration the oxygen threshold raises; in all 3 *car* mutants tested

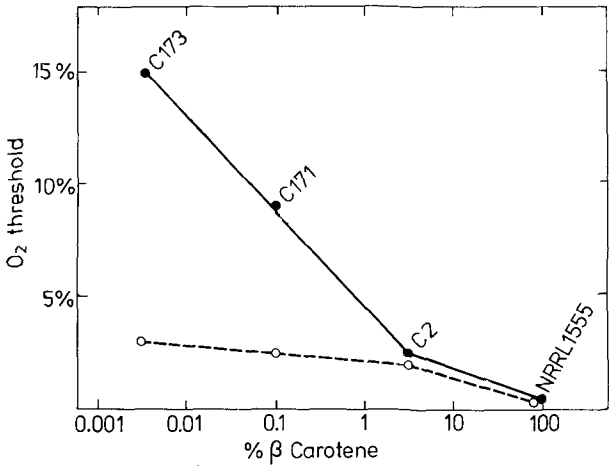


**Fig. 3.** Oxygen threshold of spph formation in dependence of β-carotene content. The indicated relative β-carotene concentrations compared to NRRL1555 have been taken from Presti et al. (1977) and Meissner and Delbrück (1968). The β-carotene concentration of C173 is below detectability and the indicated value is an upper estimate (Presti et al., 1977). The threshold values without retinal were taken from Fig. 2. ●---● without retinal; ○---○ addition of 125 μg $ml^{-1}$ retinal to the medium

**Table 3.** Oxygen consumption of *Phycomyces* in the closed system

| | | Oxygen consumption in mmol | |
|---|---|---|---|
| | | in 2 days | in 4 days |
| NRRL1555 | 18 vials | 0.5 | 2.1 |
| | 72 vials | 2.6 | 7.7 |
| C171 | 18 vials | 0.5 | 2.1 |
| | 72 vials | 2.6 | 6.7 |

Shell vials were inoculated, closed into 1 beakers and continuously kept in the dark; after 2 and 4 days samples of the atmosphere were withdrawn for oxygen determination as indicated in "Materials and Methods". The values are the average of two experiments

retinal restores spph formation to almost the wt level.

These results suggest that 1) limited oxygen concentrations are an important factor in the inhibition of sporangiophore formation in the closed system, and that 2) the supersensitivity of the *car* mutants in the closed system comes from their high oxygen threshold.

### *Oxygen Consumption in the Closed System*

For a quantitative analysis we followed the decrease

were taken out from the beakers each day and measured with an oxygen electrode. It is seen from Table 3 that there is no difference in the oxygen consumption in NRRL1555 and C171. Thus the sensitivity of *car*

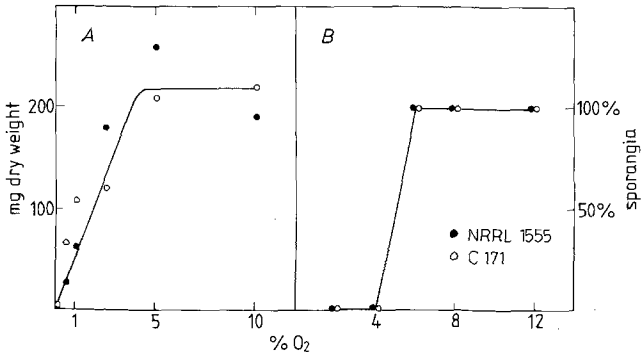


**Fig. 4 A and B.** Oxygen dependence of myceliar growth and sporangium formation; behaviour of wild type and β-carotene lacking mutant C171. **A** Potato dextrose agar plates were inoculated with ca. 500 spores and continously kept in the dark in desiccators at the indicated oxygen concentrations; the dry-weight was determined after 4 days. **B** 3 day-old stage I spph were put in dessicators in the dark and the oxygen concentration was adjusted; after 2 days the number of sporangia was determined. ● = NRRL1555; ○ = C171

mutants in the closed system is not due to enhanced oxygen consumption.

### *Oxygen Dependence of Myceliar Growth and Sporangium Formation*

Finally we wanted to see if the abnormal oxygen response of the β-carotene mutants was specific for sporangiophore formation or common to other differentiation steps such as myceliar growth and sporangium formation. Figure 4A shows the oxygen dependence of NRRL1555 and C171 in respect to myceliar growth: it is obvious that the oxygen requirement of both strains is the same; furthermore, we did not detect any difference in the oxygen threshold with respect to sporangium formation (Fig. 4B).

### *The Effect of Blue Light on Sporangiophore Initiation*

From parallel studies on photoinduction of spph in the closed system we knew that blue light overcomes the inhibitory effect of the gasatmosphere in the beaker (Galland and Russo, in press). In particular it was found that four phototropic mutants being defective in two genes had a raised threshold in the light-induced spph initiation. This observation corroborated the view that both phototropism and light-induced spph initiation are under the control of the same photoreceptor. Most surprisingly β-carotene lacking mutants displayed in this system a greatly reduced light sensitivity in comparison to the wild type. This was completely unexpected, since the same

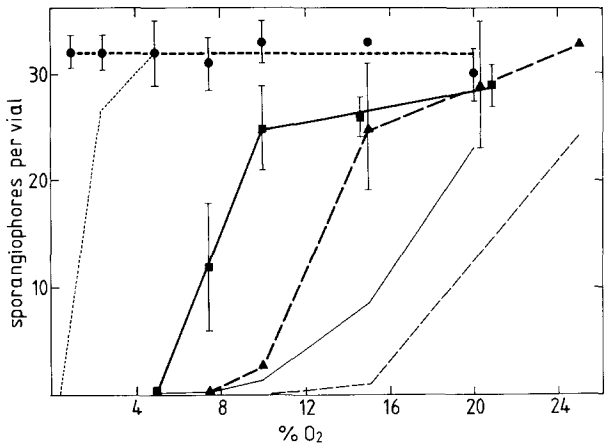


**Fig. 5.** Effect of blue light irradiation on spph formation under limiting oxygen conditions. At hour 56 the mycelia were irradiated for 1 min with blue light (20 W $m^{-2}$). Bars indicate standard error of the mean of at least 4 experiments. ●---● NRRL1555; ○—○ C171; ▲---▲ C173. The value of the unirradiated samples are indicated by the thin lines (taken from Fig. 2A)

mutants are as sensitive as wild type with respect to phototropism, thus excluding any possible involvement of β-carotene in the process of light perception in phototropism (Presti et al., 1977).

If lack of oxygen is causing the inhibition of spph initiation observed in the beaker system, one should find blue light stimulated spph initiation even under the conditions of the oxygen reconstitution experiment as in Fig. 2. Figure 5 shows that this is indeed the case for wild-type and β-carotene mutants as well. The mycelia were irradiated 56 h after inoculation with 1 min blue light with a fluence rate (20 W $m^{-2}$), which gives maximal response in the beaker system.

## Discussion

During a study of photomorphogenesis in *Phycomyces*, we found that spph formation was severely inhibited in a closed system (Russo, 1977), and in this report we have shown that a shortage of oxygen is involved in this phenomenon (Fig. 2). Surprisingly β-carotene lacking mutants are supersensitive to the atmosphere in the closed system (Fig. 1), and this led us to the observation that β-carotene lacking mutants have a raised oxygen threshold specifically in spph formation (Fig. 2). Moreover it was found that retinal, retinol, or retinol acetate suppress the supersensitivity to low oxygen tension in these mutants (Fig. 1–3). On the basis of these observations we propose that 1) *Phycomyces* synthesizes retinol which is specifically required for spph formation and that 2) in *Phycomyces* as in animal cells, retinol synthesis requires a carotene 15, 15′ dioxygenase which cleaves β-carotene, with the consumption of 1 mol oxygen per mol of β-carotene, into two molecules of retinal which is then reduced to retinol (Olson and Hayaishi, 1965; Singh and Cama, 1974; Fidge et al., 1969). The requirement of an dioxygenase for retinal synthesis in *Phycomyces* could explain the extremely high oxygen threshold of the *car* mutants.

An early report of very small amounts of retinal in *Phycomyces* has been questioned by the authors themselves and was interpreted as a spurious byproduct of β-carotene oxidation (Meissner and Delbrück, 1968). Our data suggests the synthesis of retinol in *Phycomyces*, since the defect of β-carotene lacking mutants can be overcome by retinal and retinol. Therefore, a reconsideration of this question seems to be necessary, and one should check more thoroughly whether or not the small quantities of retinal found in *Phycomyces* are artefacts.

Retinol has long been known as an important regulatory molecule in the growth and differentiation of animal cells (Moore, 1967) and its impact on the induction of specific proteins and mRNAs is well documented (Blalock and Gifford, 1977; DeLuca et al., 1971). The presence of various retinol binding proteins has been demonstrated and therefore one can envisage retinol stimulated gene activation by way of the binding proteins (Ong and Chytil, 1974; Muto et al., 1972).

Despite the key role retinol plays in differentiation in animal cells, almost nothing is known about its presence or function in plants and in fungi. In addition to the results reported here, Eslava et al. (1974) have also shown that retinol stimulates the biosynthesis of β-carotene approximately 20 fold in *Phycomyces*. This observation adds further weight to the regulatory importance of retinol in *Phycomyces*.

If our postulate, that *Phycomyces* synthesizes retinol is correct, it can easily be reconciled with the unique taxonomic position of fungi, which have metabolic features in common with animals.

We are indebted to Drs. Vennesland and Pistorius for many discussions and use of the oxygen electrode.

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