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# Identification and quantification of carotenoid pigments in aeciospores of the daisy rust fungus, *Puccinia distincta*

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**Abstract**

The yellow-orange colour of aeciospores of the daisy rust fungus, *Puccinia distincta*, was found to be due to the carotenoid pigments β,ψ-carotene (γ-carotene) and β,β-carotene (β-carotene), which were identified by means of HPLC–APCI-MS. The combined concentration of β- and γ-carotene in the aeciospores was $3.3\times10^{-15}$ mol spore$^{-1}$ or 6 mol% mol$^{-1}$ total fatty acids. This concentration is sufficient for a postulated antioxidant role of carotenoids as free radical scavengers. 

*Keywords:* *Puccinia distincta*; Pucciniaceae; Uredinales; Daisy rust; Aeciospores; HPLC–APCI-MS; Carotenoids; β,β-Carotene; β,ψ-Carotene; Fatty acids; GC–MS; Lipid droplets

## 1. Introduction

*Puccinia distincta* McAlpine (Pucciniaceae, Uredinales) is the causal agent of the current pan-European rust epidemic on wild and cultivated daisies, *Bellis perennis* L. (Preece et al., 2000). The infection is carried exclusively by wind-dispersed aeciospores; urediniospores and pycniospores are not produced, and teliospores and basidiospores are non-functional and may be relics from an ancestral species (Weber et al., 1998). Under the light-microscope, freshly harvested aeciospores of *P. distincta* show a multitude of conspicuous golden-yellow globules which have been identified as lipid droplets (Weber and Davoli, 2002; Fig. 1). These organelles are autophagocytosed by and degraded within vacuoles in the course of the ageing process of aeciospores which can be rapid, leading to senescence within 96 h (Weber and Davoli, 2002). Degradation of lipid droplets is accompanied by the loss of pigmentation from ageing aeciospores.

Lipid droplets are known to be the main site of localization of carotenoid pigments which are produced by members of many different groups of fungi (Johnson and Schroeder, 1995; Davoli and Weber, 2002). Although they have not yet been assigned a universal role in the physiology of fungi, carotenoids are known or strongly suspected to have antioxidant properties in many other organisms (Bendich and Olson, 1989; Krinsky, 1994; Edge et al., 1997), and they can protect lipid membranes from free radical oxidation in vitro (Woodall et al., 1997a,b; Rengel et al., 2000).

Among the five morphologically distinct types of spore which can be found in rust fungi, most biochemical and physiological investigations have focused on urediniospores or teliospores (Hess and Weber, 1976) whereas basidiospores, pycniospores and aeciospores have remained poorly characterized. As far as carotenoid pigments in rust spores are concerned, Lederer (1938) reported the isolation of α-, β- and γ-carotene from urediniospores of *Puccinia coronata* Corda. Later, β- and γ-carotene as well as lycopene were isolated from urediniospores of *Puccinia graminis* Persoon var. *tritici* and other cereal rusts (Irvine et al., 1954; Hougen et al., 1958), and from aeciospores of the pine rust *Cronartium fusiforme* (Valadon and Porter, 1974). Carotenoids are probably ubiquitous among rust fungi, accounting for the bright colouration of their spores (Buller, 1950; Zwetko and Pfeifhofer, 1991).

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Because of the paucity of biological data on rust aeciospores, the importance of that spore state in *P. distincta*, and the abundance of the daisy rust, we chose *P. distincta* as a source of material for our studies. The aims of the investigation reported here were firstly to identify the carotenoids present in aeciospores, and secondly to ascertain whether they are present in sufficiently high concentrations in lipid droplets of fungal spores to fulfil antioxidant functions proposed for them in other organisms.

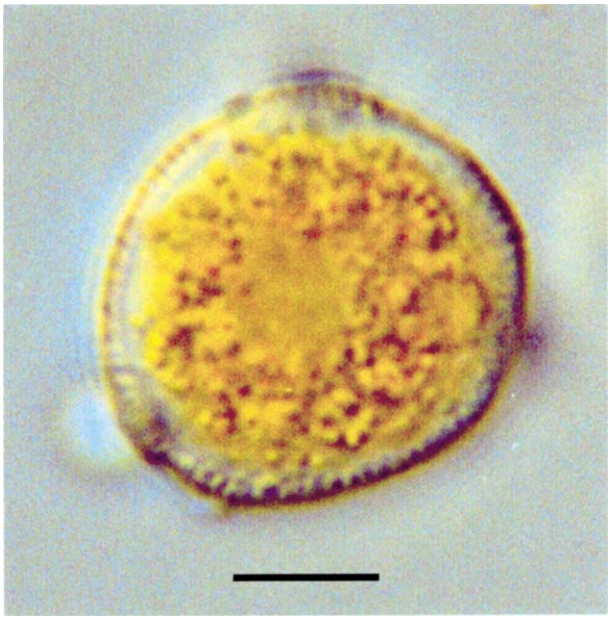

Fig. 1. Photomicrograph of a freshly-harvested aeciospore of *Puccinia distincta*. The spore is filled with numerous lipid droplets which appear yellow due to their high carotenoid content. Bar = 5 μm.

## 2. Results and discussion

Four independent samples of aeciospores (2.5–$4.8\times10^7$ spores per sample) were analysed in detail. In each of these, as well as in several preliminary samples, two pigments were detected by reversed-phase HPLC analysis (Fig. 2) and were identified as β,ψ-carotene (γ-carotene) and β,β-carotene (β-carotene) on the basis of their UV/visible spectra ($\lambda_{max}$ 460 and 452 nm, respectively). Confirmation of the identity of both pigments was provided by HPLC–APCI-MS analysis in the negative ionization mode. The expected molecular ion $M^-$ at $m/z$ 536 was obtained for both compounds as the base peak (van Breemen et al., 1996), thus confirming the structure deduced from UV/visible spectroscopy.

The quantification of the two carotenoids from aeciospores was performed by HPLC using a β-carotene commercial standard. For this purpose, four different samples of *P. distincta* aeciospores were processed and analysed by HPLC. γ-Carotene represented the main pigment in each sample, accounting for 66±7% of the total carotenoid content. This value agrees closely with

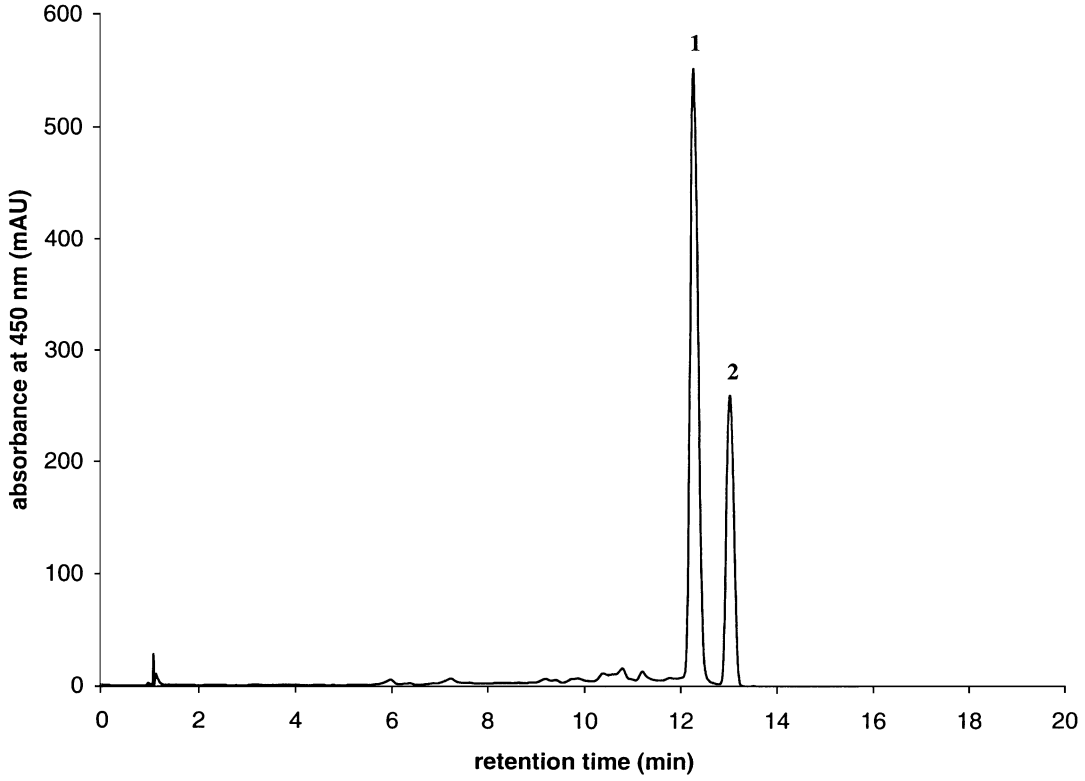


Fig. 2. Pigment profile of *Puccinia distincta* aeciospores by reversed-phase HPLC (see Experimental for conditions). **1**: β,ψ-carotene; **2**: β,β-carotene.

the data of Valadon and Porter (1974) on aeciospores of *Cronartium fusiforme*. The average content of carotenoids per aeciospore of *P. distincta* was found to be 1.78±0.42 pg/aeciospore, corresponding to 3.31±0.78 fmol/aeciospore. The same aeciospore extracts were also processed to evaluate their lipid content by means of GC–MS analysis of fatty acid methyl esters, following a protocol modified from Perrier et al. (1995). (*Z*,*Z*)-9,12-Octadecadienoic, (*Z*)-9-octadecenoic, hexadecanoic and octadecanoic acid (linoleic, oleic, palmitic and stearic acid, respectively) accounted for ca. 90% of the total lipid content. This fatty acid composition is in fair agreement with data on aeciospores of *P. graminis* (Tulloch and Ledingham, 1962). In contrast, tetra-, penta- and heptadecanoic as well as eicosanoic and docosanoic acid were present only in minor amounts. The total fatty acid content per aeciospore was found to be 16.5±1.6 pg/aeciospore, corresponding to 57.7±5.7 fmol/aeciospore. Therefore, a concentration of 6 mol% of carotenoids relative to the total fatty acid content was calculated.

Such a value is six times higher than the concentrations of β-carotene or other carotenoids which were sufficient to inhibit lipid peroxidation in liposomes containing phosphatidylcholine (Woodall et al., 1997b). At a concentration of 1 mol% relative to phosphatidylcholine and in the presence of peroxyl radicals, carotenoids reduced the rate of formation of lipid peroxidation products, thus acting as effective lipid-soluble antioxidants. Given that fatty acids are stored mainly as triacylglycerides and, like carotenoids, are located in lipid droplets (Lösel, 1988), our results indicate that the combined concentration of β- and γ-carotene would be sufficient to fulfil a similar role in protecting the lipid storage reserves of fungal spores. Future work should test the hypothesis of an antioxidant function of carotenoids in fungal spores by comparing the viability of wild-type aeciospores with that of colourless carotenoid-deficient spores of mutant strains under conditions of oxidative stress. Such mutants have been described for several rust species, but so far they have been investigated merely for their tolerance of UV light, which seems to be reduced (Zwetko and Pfeifhofer, 1991). Evidence for an antioxidant role of carotenoids in the physiology of fungi comes from studies on pigmented yeasts which show an enhanced synthesis of carotenoids under conditions of oxidative stress, and a correspondingly increased resistance to singlet oxygen radicals (Sakaki et al., 2000).

## 3. Conclusions

Carotenoid pigments were found to be responsible for the yellow pigmentation of *P. distincta* aeciospores. γ-Carotene and β-carotene were identified in aeciospore extracts by means of HPLC–APCI-MS analysis and their concentration was also determined by quantitative HPLC. Each aeciospore contained about 2 pg of the two carotenoids, with γ-carotene accounting for 66%.

Although the function of carotenoids in fungal spores has not yet been proven experimentally, these pigments are likely to play an important role as antioxidants also in these organisms, especially in the protection of sensitive biological molecules from reactive oxygen species, such as free radicals. The localization of sufficient amounts of γ-carotene and β-carotene within lipid droplets in aeciospores of *P. distincta* hints at a possible lipid-protecting function.

## 4. Experimental

### 4.1. Pigment extraction

Leaves of wild *Bellis perennis* bearing fresh aecial lesions were collected from lawns in the vicinity of the University of Kaiserslautern, Germany, from April to September 2001. Voucher specimens have been deposited in the Herbarium, Dept. of Biotechnology, University of Kaiserslautern, ref. no. RW01002, and are available on request. Aeciospores were harvested by gently scraping the aecial infections with a razor blade in the presence of a few drops of distd. water and collected as an orange spore suspension. In addition, scraped leaves were soaked in water and briefly sonicated (10 s). The aeciospore suspensions were pooled, centrifuged at 10 000 rpm (7800 *g*) for 10 min, resuspended in water (20 ml) and counted using a haemocytometer chamber. Four different samples were collected and processed. Each aeciospore suspension was frozen at −20 °C, homogenized with an X-press (AB Biox, Sweden), thawed and extracted with EtOAc (2×20 ml) and cyclohexane (2×20 ml) until disappearance of any pigmentation from the aqueous phase. Where necessary, centrifugation at 10 000 rpm for 5 min was performed to separate the two phases. The yellow organic phases were pooled, dried over $Na_2SO_4$, filtered and rotary evaporated at rt to afford a dark yellow-orange residue which was redissolved in acetone and subjected to HPLC analysis. All operations were carried out under subdued light to avoid pigment degradation.

### 4.2. Pigment analysis

HPLC analyses (Hewlett Packard 1090 Series II Liquid Chromatograph) were performed on a LiChrospher® 100 RP-18 (5 μm; 125×4.0 mm) column, using a linear gradient of acetonitrile/$H_2O$/HCOOH (86:10:4 v/v/v; solvent A) and EtOAc/HCOOH (96:4 v/v; solvent B): 0 min, 100% A; 20 min, 100% B; flow rate 1.0 ml/min; detection at 450 nm. The two pigments detected in

all samples ($t_R$ 12.2 and 13.0 min) were identified as β, ψ-carotene (UV: $\lambda_{max}$ 430, 460, 489 nm) and β,β-carotene ($\lambda_{max}$ 425, 452, 478 nm), respectively. In order to confirm their identity, samples were partially purified by preparative TLC (Silica gel $60_{F254}$, Merck), using cyclohexane/EtOAc 95:5 as the eluant; the bright orange band with $R_f$ 0.70 was scraped off, dissolved in cyclohexane and acetone, and finally subjected to HPLC–APCI-MS analysis (Hewlett Packard Series 1100LC-MSD Liquid Chromatograph-Mass Spectrometer) in the negative ionization mode (NI), with the fragmentor voltage set at 70 or 100 V and using similar HPLC conditions.

Quantitative HPLC determination of β-carotene in the aeciospore extracts was achieved using a synthetic standard (Merck), whereas for γ-carotene appropriate correction factors were applied, using the coefficients published by Britton (1995). For each sample, the amount of total carotenoids per aeciospore was calculated and the data were then averaged. The above reported values are expressed as the average of four samples ± standard deviation.

### 4.3. Fatty acids analysis

After HPLC determination of carotenoids, the crude extracts were concentrated and assayed for fatty acid content by means of GC–MS technique (as methyl esters). Each sample was dissolved in 500 μl of *n*-heptane and 200 μl were treated with 20 μl of a freshly prepared solution of sodium methoxide in MeOH. As internal standard for quantitative determination, 10-undecenoic acid methyl ester was used, synthesized by Fischer esterification of commercial undecylenic acid (Jeffery and Vogel, 1948; Jordan and Swern, 1949); 5 μl of a 10 μg/μl stock solution in *n*-heptane were added to each sample prior to transesterification, and the mixture was vigorously stirred and left to react at rt for at least 1 h. The mixture of fatty acid methyl esters thus formed was analyzed by gas chromatography on a HP-5MS column (30 m×0.25 mm, 0.25 μm film thickness) coated with crosslinked (5%)-diphenyl–(95%)-dimethylpolysiloxane copolimer, using a Hewlett Packard 5890 Series II chromatograph coupled to a HP5989A mass spectrometer (EI, 70 eV) and identified on the basis of their fragmentation pattern by direct comparison with the database library. Injector and detector temperatures were 250 and 280 °C, respectively; the column temperature was kept at 120 °C for 1 min, raised to 220 °C at a rate of 20 °C/min and then up to 250 °C at 5 °C/min, and finally maintained at 250 °C for 5 min.

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