An Improved Procedure for the Quantitative Estimation of the Rust Fungus in Infected Plant Tissue

Gertrud Wiese, Doris Hugo-Wissemann, and Hans J. Grambow

Institut für Biologie III (Pflanzenphysiologie), Technische Hochschule, Worringer Weg, D-5100 Aachen, Bundesrepublik Deutschland

Z. Naturforsch. **41c,** 1127–1130 (1986); received July 17, 1986

Rust Fungus, Chitin, N-Acetylglucosamine

A quantitative estimation of the rust fungus in infected wheat leaves was possible following enzymatic hydrolysis of the chitin and colorimetric determination of the N-acetylglucosamine released. The apparently complex cell wall structure of the fungal structures made it necessary, however, to use an enzyme mixture of chitinase and cellulase in order to make accessible the chitin of the cell wall for digestion by chitinase. In applying this method the measurement is not appreciably influenced by already formed urediospores as would otherwise be the case were the chitin to be determined on the basis of glucosamine after chemical hydrolysis.

Introduction

A number of different techniques has been recommended for the quantitative estimation of pathogenic fungi in host tissue (see [1], and literature cited therein). Among others, serological estimations using ELISA or the chemical determination of the chitin content have been employed. The ELISA technique offers the advantage of a very high sensitivity and possibly of differentiating between fungi growing on the same leaf [2]. However, some restrictions can arise because the surface carbohydrates and, consequently, antigenic determinants of the wall may change during the course of fungal infection. Thus, the increase or decrease in the selected antigen, rather than the growth of the fungus, is that which is measured in such a case [3].

For chemical estimations of fungi ergosterol has been recommended as a measure of fungal growth [4]. The assay for chitin developed by Ride and Drysdale [5] has been widely used to measure fungal biomass in infected plant tissue. This assay is based on the alkaline deacetylation of chitin to chitosan, the glucosamine residues of which are susceptible to deamination with nitrous acid. This yields an aldehyde which is determined colorimetrically with 3-methyl-2-benzothiazolone hydrazone. In a more recent study it has been stressed that this chitin assay does not detect changes in vegetative growth of rust fungi but only a reactive component, probably glucosamine, which is present in mature and developing urediospores [6]. Similarly, a different chitin assay based upon the acidic hydrolysis of plant tissue and the ninhydrin reaction with glucosamine does not exclude such a possibility [7].

Because of these restrictions we tried to develop a technique for chitin estimation which is not interfered with by spore materials such as glucosamine which occur in high amounts in mature urediospores [8–10]. A promising approach appeared to be the estimation of chitin by the specific colorimetric detection of N-acetylglucosamine obtained after enzymatic hydrolysis of chitin [11]. This approach, in fact, proved to be successful when care was taken to ensure that fungal wall structures in the host-parasite complex were completely hydrolyzed by the use of suitable enzyme mixtures.

Materials and Methods

Inoculation of susceptible wheat leaves (*Triticum aestivum cv.* Marquis and *Triticum compactum cv.* Little Club) with urediospores of *Puccinia graminis f. sp. tritici*, race 32, and of oat leaves (*Avena sativa cv.* Pc40) with urediospores of *Puccinia coronata avenae*, race HKR KoII, was carried out as described elsewhere (Wiese and Grambow, submitted).

Homogenization and hydrolysis of leaf material: Inoculated wheat leaves (0.5 g) were frozen in liquid nitrogen, powdered in a mortar, suspended in acetate buffer (2.4 ml, 0.08 M, pH 5.0) in centrifuge tubes and heated for 10 min in order to inactivate enzymes. After cooling, cellulase (0.5 ml, 3% aqueous solution of Onozuka R10, isolated from *Trichoderma viride*), and chitinase (0.3 units, isolated from *Streptomyces griseus*) were added. Chloroform (50 µl) was added in order to prevent microbial contamination and the samples were then sonicated for 2 min, sealed and incubated at 37 °C for 48 h. Following centrifugation, aliquots of the clear supernatant (usually 50 or 100 µl) were used for the colorimetric estimation of N-acetylglucosamine. Preparations obtained from healthy wheat leaves were used as a reference.

Reprint requests to Prof. Dr. H. J. Grambow.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/1100–1127 $ 01.30/0

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

The incubation period (48 h) proved to be sufficient since no higher yield of N-acetylglucosamine was obtained after prolonged incubation periods.

Treatment of urediospores and mycelium: Urediospores of *Puccinia graminis tritici* (Pgt) or *Puccinia coronata avenae* (Pca) were suspended in buffer (2.5 ml) and treated in the same manner as the leaf material.

Small samples of axenically-grown mycelium were treated likewise. The mycelium was cultured according to Kuck and Reisener [12] with the following modifications: small amounts of acetone (1‰) were added in order to stimulate vegetative growth (Poppe, personal communication), and the mycelium was cultured on 5 µm nuclepore membranes placed on the nutrient agar in order to facilitate harvesting of the mycelium.

Estimation of N-acetylglucosamine was performed according to Reissig *et al.* [11]. The method is specific for acetylated aminosugars, which produce a glucoxazoline after heating under alkaline conditions [13]. After reaction with p-dimethylaminobenzaldehyde, a pigment is produced which can be measured at 585 nm.

Chemicals: Cellulase Onozuka R10 was obtained from Serva, FRG, and the chitinase was from Sigma.

Results and Discussion

Table I clearly shows that high recoveries were obtained in the determination of chitin if the leaf segments were treated not only with chitinase, but also simultaneously with cellulase.

Control experiments showed that neither in non-infected leaves, nor in the enzyme preparations used, were substances contained which interfered with the test. Furthermore, it became evident that the cellulase preparation used contained a certain chitinase activity (probably this was an accompanying enzyme; it was not further investigated however). The latter was confirmed by the attempt to hydrolyse colloidal chitin. In this case there was no significant difference in activity between pure chitinase and the chitinase/cellulase mixture. The cellulase preparation alone however already contained (when introduced at the concentrations used here) about 40% of the chitinase activity (not shown in table).

In order to be able to assess to what extent the urediospores formed in the leaves affected the measurement, spores from wheat stem rust and oat crown rust were treated with the enzyme mixture. The results show that in applying this method only very little positively-reacting material is released from spores.

In additional experiments the chitin content was similarly determined in axenically-cultivated mycelium. Surprisingly it transpired that in these samples too a chitin hydrolysis took place only with simultaneous action of chitinase and cellulase. This showed that the effect of the cellulase preparation apparently does not result from the fungal mycelium being more accessible for chitinase by the hydrolysis of plant structures. Rather, cellulase-activity leads to better digestion of the fungal cell wall. According to the accounts given by the producers, the enzyme preparation used contains hemicellulases as accom-

Table I. N-acetylglucosamine content of healthy and inoculated leaves (10 days after inoculation), axenically grown mycelium, and urediospores of *Puccinia graminis tritici* (Pgt) or *Puccinia coronata avenae* (Pca) after treatment with cellulase, chitinase, or cellulase + chitinase.

Sample	Treatment	N-acetylglucosamine [µg/g fr. wt.]
Inoculated leaves	chitinase	20
Inoculated leaves	cellulase	308
Inoculated leaves	chitinase + cellulase	834
Healthy leaves	chitinase + cellulase	21
Mycelium	chitinase	70
Mycelium	chitinase + cellulase	5220
Urediospores (Pgt)	chitinase + cellulase	219
Urediospores (Pca)	chitinase + cellulase	261

panying enzymes and degrades mannans, xylans, galactomannans and other polymeric carbohydrates. It should be taken into consideration that, most likely, these activities are involved in the effect.

The observation that only the use of the enzyme mixture leads to a satisfactory cleavage of chitin suggests that the fungal cell wall presents a highly complex structure. Only simultaneous digestion of different cell components leads to an optimal recovery of the monomers. This hypothesis is supported by the observation of Joppien [14]: in germtubes of the wheat rust freed of lipids there is a considerable increase in the release of glucose by glucanase treatment following pretreatment with chitinase. Ride and Drysdale [15] reported that commercial chitinase gave low yields of acetylglucosamine from *Fusarium oxysporum f. lycopersici* cell walls unless used with snail digestive juice. The use of this mixture however proved lengthy and expensive. A direct indication of the complex wall structure of intercellular hyphae of the rust fungus as well as haustorial mother cells was obtained using cytochemical studies: chitin was detected in the innermost layers of the fungal structures [16]. In this context it should again be mentioned that the structures of the rust fungus, including that of the hyphae, are covered by a complex coat of surface carbohydrates [3].

The observation that an enzyme mixture is necessary for the digestion of the fungal cell wall furthermore permits the speculation that the apparently complex cell wall structure may exert a protective function and impedes digestion of the fungal structures by extracellular enzymes of the host plant. With regard to chitin, the hypothesis has already been formulated that pathogen-induced chitinase in host plants plays a defensive role against pathogenic fungi [17]. At least in the case of rust fungi, it can be assumed from the data presented here that to practise such a defensive reaction the simultaneous appearance of additional polysaccharide-degrading enzymes should be necessary.

In the present study the fungal growth could be measured by determination of chitin on densely infected wheat leaves (ca. 100 pustules/cm^2) beginning with the third to fourth day after inoculation (Fig. 1). This result is well supported by microscopic investigations: whereas the colonies only increase very slowly during the first three days, from the fourth day onward there begins a massive spreading. The first mature spores were formed in those systems examined six days after inoculation and one day later the majority of the colonies achieved sporulation.

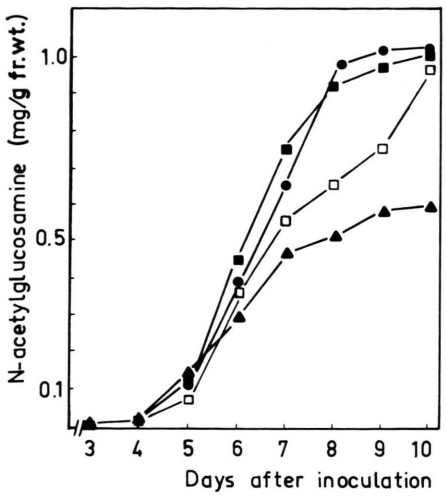

Fig. 1. Estimation of N-acetylglucosamine content, representing fungal chitin of stem rust infected wheat leaves, after treatment of homogenates with a mixture of cellulase and chitinase.
●——● *Triticum compactum cv.* Little Club infected with *Puccinia graminis tritici*, race 32.
■——■ *Triticum aestivum cv.* Marquis infected with *Puccinia graminis tritici*, race 32.
□——□ *Triticum aestivum cv.* Marquis (same as above); the depression in the N-acetylglucosamine content between day 6 and 8 was observed occasionally in some experiments; eventually, it may reflect some irregularities in the growth of the fungus; the true reason, however, is not known.
▲——▲ *Avena sativa cv.* Pc40, infected with *Puccinia coronata avenae*, race HKR KoII.

The results show that the chitin content exhibits a continual increase but not that abrupt increase at the onset of sporulation as reported in earlier studies based on glucosamine determination. Thus we assume that the data obtained in the present study more precisely represent the chitin content *i.e.* the vegetative growth, of the mycelium in the leaf.

Acknowledgements

The authors thank Prof. K. Mendgen, Konstanz, for a gift of *Puccinia coronata avenae* and seeds of *Avena sativa* and Mrs. G. E. Grambow for technical assistance and preparation of the manuscript.

[1] K. Mendgen, in: Microbiology of the Phyllosphere (N. J. Fokkema and I. v. d. Heuvel, eds.), p. 50, Cambridge University Press, Cambridge 1986.
[2] R. Casper and K. Mendgen, Phytopathol. Z. **94,** 89 (1979).
[3] K. Mendgen, M. Lange, and K. Bretschneider, Arch. Microbiol. **140,** 307 (1985).
[4] L. M. Seitz, D. B. Sauer, R. Burroughs, H. E. Mohr, and J. D. Hubbard, Phytopathology **69,** 1202 (1979).
[5] J. P. Ride and R. B. Drysdale, Physiol. Plant Pathol. **2,** 7 (1972).
[6] S. G. W. Kaminsky and M. C. Heath, Can. J. Bot. **60,** 2575 (1982).
[7] S. Mayama, D. W. Rehfeld, and J. M. Daly, Physiol. Plant Pathol. **7,** 243 (1975).
[8] L. A. Hadwiger and R. F. Line, Physiol. Plant Pathol. **19,** 249 (1981).
[9] S. Joppien, A. Burger, and H. J. Reisener, Arch. Mikrobiol. **82,** 337 (1972).
[10] P. Trocha, J. M. Daly, and R. J. Langenbach, Plant Physiol. **53,** 519 (1974).
[11] J. L. Reissig, J. L. Strominger, and L. F. Leloir, J. Biol. Chem. **217,** 959 (1955).
[12] K. H. Kuck and H. J. Reisener, Physiol. Plant Pathol. **27,** 259 (1985).
[13] T. White, J. Chem. Soc. **1940,** 428.
[14] S. Joppien, Ph. D. Thesis, RWTH Aachen (1975).
[15] J. P. Ride and R. B. Drysdale, Physiol. Plant Pathol. **1,** 409 (1971).
[16] J. Chong, D. E. Harder, and R. Rohringer, Can. J. Bot. **63,** 1713 (1985).
[17] J. P. Métraux and T. Boller, Physiol. Molec. Plant Pathol. **28,** 161 (1986).