Phytochemistry, 1976, Vol. 15, pp. 953–954. Pergamon Press. Printed in England.

GLYCINEBETAINE AND CHOLINE IN WHEAT: DISTRIBUTION AND RELATION TO INFECTION BY *FUSARIUM GRAMINEARUM*

RAYMOND B. PEARCE*‡, RICHARD N. STRANGE* and HARRY SMITH†
*Department of Botany and Microbiology, University College, Gower Street, London WC1E 6BT
†Department of Microbiology, P.O. Box 363, The University, Birmingham B15 2TT, England

(*Revised received* 24 *December* 1975)

Key Word Index—*Triticum aestivum*; Gramineae; *Fusarium graminearum*; fungi imperfecti; wheat; glycinebetaine; choline; quaternary nitrogen compounds; periodide assay.

Abstract—Glycinebetaine and choline, which stimulate the growth of *Fusarium graminearum* both *in vitro* and *in vivo*, are present in higher concentrations in wheat anthers relative to any other organ as measured both by the periodide assay and a bioassay. This finding provides an explanation for the onset of greatly increased susceptibility of wheat to *F. graminearum* at anthesis.

INTRODUCTION

Wheat is very susceptible to infection by *Fusarium graminearum* at anthesis but resistant if anthers are removed. Growth of the fungus on anthers *in vivo* is luxuriant, suggesting that these organs are richer in nutrients required by the parasite than other parts of the plant. Further evidence for this view was obtained when the fungus was grown *in vitro* on a medium supplemented with extracts of wheat organs. All extracts were stimulatory to some extent, but those from anthers contained more stimulant than those from any other organ and also caused virulence enhancement [1]. The stimulatory material was purified and yielded two crystalline compounds which were identified by MS as glycinebetaine and choline [2]. As will be reported elsewhere, these compounds stimulate the infection of wheat by *F. graminearum* and the growth *in vitro* of other cereal parasites of the genus *Fusarium*. The growth *in vitro* of the maize parasite, *Diplodia maydis*, is also stimulated but that of several other fungi is not [3].

The satisfaction of nutritional requirements of parasites by their hosts has been postulated as one explanation for the high degree of specificity often found in host-parasite relations [4], and considerable evidence has been obtained for it in a number of systems [5–8]. In order to determine whether such a role may be ascribed to glycinebetaine and choline, it was necessary to estimate accurately their concentration in plant tissue. For this purpose the periodide method [9] seemed the most appropriate, since, unlike other methods, it enabled both compounds to be measured without prior separation. The experiments reported here were designed to test the sensitivity and specificity of the assay, to establish the distribution of glycinebetaine and choline in wheat, and to compare these results with those from a bioassay involving the growth stimulation of *F. graminearum* [1, 10].

RESULTS AND DISCUSSION

Sensitivity and specifity

Authentic glycinebetaine and choline could be measured independently by the periodide assay in mixed samples, since both compounds are precipitated as their periodides in acid solution, but only choline at pH 7.0. The useful lower limits of sensitivity in the 0.5 ml samples are 0.20 μm glycinebetaine and 0.04 μM choline. Both compounds were extracted efficiently by the method described since material supplemented with either compound yielded results within $\pm$ 8% of theoretical. PLC of crude extracts revealed a third compound besides glycinebetaine and choline on exposure of the plate to I_2 vapour which was identified by co-chromatography and MS as trigonelline. This compound formed a periodide in M HCl, but not in the phosphate buffer. It contributed less than 10% to the values recorded for betaine for all wheat organs except glumes, where it was 15%. Of the combined forms of choline tested, only lecithin and glycerophosphorylcholine can cause interference. Lecithin was positive in the assay but was removed by the extraction procedure whereas glycerophosphorylcholine was negative in the assay but was partially hydrolysed to choline during extraction. This compound has been reported as only a minor constituent of plant material [11] and was therefore unlikely to be the cause of substantial error.

Comparison of the periodide assay with the fungal bioassay of crude extracts

Choline is a more potent stimulant of *F. graminearum* than glycinebetaine (2201 and 709 units of stimulant activity μM respectively [3]). Using these conversion factors and the data from the periodide assay, the stimulant activity in extracts caused by choline and glycinebetaine was compared with that recorded directly in the bioassay

‡ Present address: Department of Microbiology, P.O. Box 363, The University, Birmingham, B15 2TT, England.

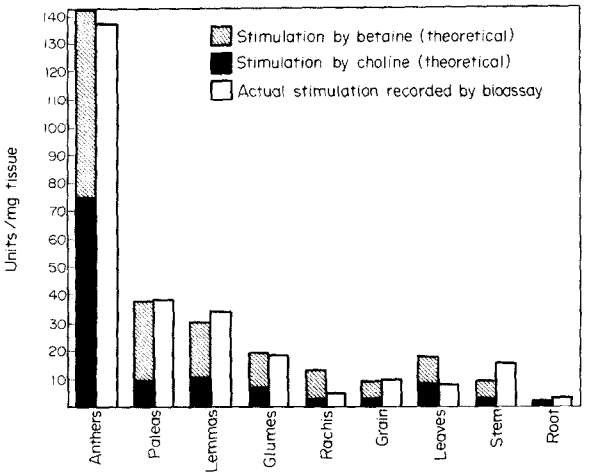

Fig. 1. Glycinebetaine and choline levels of wheat organs estimated by the periodide assay and expressed as units of stimulant activity/mg. compared with actual activity recorded by direct bioassay. 1 unit of activity is equivalent to 1.43 nmol glycinebetaine and 0.454 nmol choline.

(Fig. 1). In samples from the rachis and leaves, stimulant activity was lower than that predicted by the periodide assay, possibly owing to amorphous material which was precipitated by the KI/I_2 reagent but was insoluble in dichloroethane. This material appeared to trap iodine which was then released into the dichloroethane solution, thus contributing to the absorbance value. Otherwise, particularly in view of the variation encountered with the bioassay [10], reasonable agreement was obtained between the two assays. More importantly, the higher concentrations of choline and betaine in anthers relative to other organs provide an explanation for the onset of susceptibility of wheat to *F. graminearum* at anthesis.

EXPERIMENTAL

Plant material. Spring wheat (*Triticum aestivum*) cvs. Marquis and Troll were grown in a greenhouse (16 hr photoperiod, minimum temp. c. 23°, in soil) or in a controlled environment cabinet (16 hr photoperiod, 10,800 lx, 20°, in sand fed with nutrient salts (Long Ashton formulation [12])).

Preparation of extracts. Wheat organs (0.2–1.5 g) and sand (0.5 g) were ground in a mortar with 20 ml MeOH. After centrifugation the residue was re-extracted with MeOH(× 3). The extract was evaporated to dryness and the residue dissolved in an Et_2O–HCl (0.1 M) (1:1) (3 × 20 ml). The phases were allowed to separate, and the combined aq. phases were extracted with Et_2O (10 ml). Combined Et_2O phases (including that from the last extraction) were extracted (× 4) with HCl (0.1 M, 10 ml). The aq. phases (combined) were evaporated to dryness and residue dissolved in H_2O (200–500 mg fr. wt material extracted/ml.).

Bulk extracts (10–50 g) were prepared similarly except that a blender was used, with 4 × 250 ml aliquots MeOH. Partitioning procedures were carried out using 50 ml aliquots of each phase, the final extracts being made up to 4 g fr. wt/ml.

Assay method. This was based on the method of Wall *et al.* [9]. Extracts were diluted 1:1 with HCl (2 M) for the precipitation of glycinebetaine and choline periodides, and with KPi buffer (0.2 M, pH 7.0) for the precipitation of choline periodide alone. After adjustment of the pH, any precipitate was removed by centrifugation. Aliquots (0.5 ml) of the diluted extracts were transferred to 12 ml tapered glass centrifuge tubes, and held at 0° (10 min) to equilibrate. 0.2 ml cold KI/I_2 reagent (15.7 g I_2, 20 g KI, made up to 100 ml (H_2O) was added to each tube with gentle shaking. Tubes were held at 0° (80 min), and scratched occasionally with a glass rod to ensure complete crystallisation of the periodides. After centrifugation (2000 g, 5 min) the supernatant was thoroughly removed by aspiration, the precipitate dissolved in 1,2-dichloroethane (10 ml), and the absorbance read at 365 nm (10 mm path length). Standard solutions of glycinebetaine and choline Cl were used for the preparation of standard curves and to confirm the precipitation of both compounds in M HCl, but only choline in KPi buffer. Standards of both compounds were included in all assays of plant extracts.

Test for efficiency of extraction. Leaf samples (1 g) were supplemented with 1 mg each of glycinebetaine and choline Cl. dissolved in 1.0 ml H_2O prior to extraction.

Specificity of assay. Bulk extracts were fractionated by PLC, using the methods of Eneroth and Lindstedt [13]. Plates (Si gel, 1 mm layer thickness) were developed in 1 dimension in MeOH–Me_2CO–HCl (11 M) (90:10:4) or in 2 dimensions using this solvent after MeOH–NH_3 (sg. 0.880) (75:25). Quaternary nitrogen compounds were visualised with I_2 vapour. The identity of trigonelline was proved by co-chromatography with an authentic sample (in 2 dimensions) on PLC, and by MS. Extract samples (0.8 g fr. wt equivalent) were separated on PLC in 1 dimension, the quaternary nitrogen compound spots visualised, removed from the plates, and extracted with 4 × 5 ml aliquots of MeOH. This procedure gave better than 93% recovery of pure glycinebetaine, choline and trigonelline from the stationary phase. The extracts were dried, made up in H_2O and assayed in HCl.

Behaviour of choline compounds. Leaves (0.5 g) were supplemented with sulphurylcholine, phosphorylcholine Cl (Ca Salt) and glycerophosphorylcholine (0.3 ml), aqueous soln 10 mg/ml) or lecithin (0.3 ml, MeOH soln, 20 mg/ml), prior to extraction and assay. Samples of the compounds were also assayed directly.

Bioassay of extracts with F. graminearum. Bioassays were performed as previously described [2].

Acknowledgements—We wish to thank the Science Research Council for financial support and Mrs. M. Gough for technical assistance.

REFERENCES

1. Strange, R. N. and Smith, H. (1971) *Physiol. Plant Path.* **1,** 141.
2. Strange, R. N., Majer, J. R. and Smith, H. (1974) *Physiol. Plant Path.* **4,** 277.
3. Strange, R. N. and Smith, H. Unpublished data.
4. Garber, E. D. (1956) *Am. Nat.* **90,** 183.
5. Lukezic, F. L. and De Vay, J. E. (1964) *Phytopathology* **54,** 697.
6. Smith, H., Williams, A. E., Pearce, J. H., Keppie, J., Harris-Smith, P. W., Fitzgeorge, R. B. and Witt, K. (1962) *Nature* **193,** 47.
7. Burrows, T. W. and Gillet, W. A. (1971) *Nature* **229,** 51.
8. Coplin, D. L., Sequeira, L. and Hanson, R. S. (1974) *Can. J. Microbiol.* **20,** 519.
9. Wall, J. S., Christianson, D. D., Dimler, R. J. and Senti, F. R. (1960) *Anal. Chem.* **37,** 870.
10. Strange, R. N. and Smith, H. (1971) *Trans. Brit. Mycol. Soc.* **56,** 485.
11. Toyosawa, I. and Nishimoto, U. (1967) *Agr. Biol. Chem.* **31,** 27.
12. Hewitt, E. J. (1966) *Sand and Water Culture Methods used in the Study of Plant Nutrition.* 2nd Ed. p. 430. Commonwealth Agricultural Bureaux.
13. Eneroth, P. and Lindstedt, G. (1965) *Anal. Biochem.* **10,** 479.