Reprint from: Neth. J. Pl. Path. 74 (1968 Suppl. 1) 157–162

A fungitoxic substance extracted from tulips and its possible role as a protectant against disease

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Abstract

A specialized strain of *Fusarium oxysporum* is the cause of a disease important in tulip bulb cultivation. From observations in the field it was concluded that the fungus is not able to penetrate into the tulip bulb during the greater part of the growing season. A protecting barrier was demonstrated to be present in the white skin. From extracts of this white skin (and from other parts of the tulip plant) a fungitoxic substance was isolated, which was identified as α-methylene butyrolactone. A possible precursor of this lactone has been isolated which, under certain conditions, yields the lactone in vitro. The implications of the presence of the fungitoxic substance in extracts made from different parts of the plant in relation to the susceptibility to *Fusarium* are discussed.

Introduction

A specialized strain of *Fusarium oxysporum* Schlecht. is the cause of a disease of great economic importance in tulip bulb cultivation. Tulip bulbs are planted in autumn and harvested in July of the following year. During this period – and mainly after the plant has flowered in April/May – buds standing in the axils of the scales of the planted bulb grow out into new daughter bulbs. Each bulb consists of several concentric layers of white and fleshy scales, which are implanted on a basal plate. During outgrowth of the young bulb its outermost scale has the same appearance as the other scales: it is white and fleshy and consists of living cells rich in sugars and starch. At the end of the growth period, when the leaves and the stem wither, the contents of this outer scale disappear and its cells die, and finally it turns brown and papery, while the underlying scales remain unaltered. For the sake of convenience the name "white skin" has been introduced for this part, which turns into the "brown skin" and which encloses the "fleshy bulb scales".

Results

Observations on infection

Under conditions prevailing in The Netherlands *F. oxysporum* invades the bulb only occasionally through the roots and the basal plate. Only when circumstances are very favourable for the fungus (e.g. high soil temperature in the glasshouse during forcing

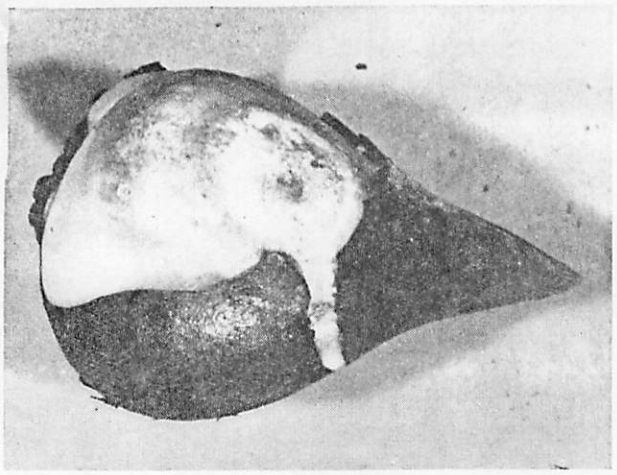

Fig. 1. Tulip bulb several weeks after harvest. The brown skin, which itself is unaffected, has been removed partly to show the symptoms of infection by *F. oxysporum* in the underlying outer fleshy scale, initiating from two points on the side of the bulb.

in winter) this type of attack is found more often. Under these conditions the lowest part of the plant stem can also be invaded by the fungus. In general, however, it causes nearly exclusively a dry rot of the fleshy bulb scales, symptoms of which mainly become evident during storage after lifting (Bergman, 1965). Symptoms of infection by *F. oxysporum* are never found in the white or in the brown skin (Fig. 1). Some years ago it has been proved that in The Netherlands the bulbs are infected in the field nearly exclusively during the last weeks before harvesting of the bulbs.
Though soil temperature has been found to influence the infection in the field, it was also found that this factor alone was not sufficient to explain why the young bulb is infected in general only during the last stage of its development (Bergman, 1966). It seemed as if a barrier is protecting the young bulb during the greater part of its developmental period, which barrier only can be broken by the fungus during the last weeks before lifting. Since the white skin turns brown and papery exactly in the same period, it seemed plausible that the supposed barrier was located in the white and living skin.

Isolation of a fungitoxic substance
Extracts of white skin were made by grinding them in Sørensen phosphate buffer pH 7.5. Growth of *F. oxysporum* was retarded on potato dextrose agar mixed with partly purified extracts. By making dilution series of the extracts in potato dextrose agar it was possible to compare the relative amounts of the substances in different tissues or in the same tissue at different moments during plant and bulb development.
In the highest concentration of the dilution series of Fig. 2 the active principle proved to be fungicidal. This lethal concentration was about 100 ppm (Bergman et al., 1967). Complete purification of the substance was obtained by column-chromatography and steam distillation. It has been identified as α-methylene butyrolactone[1]. The amount of

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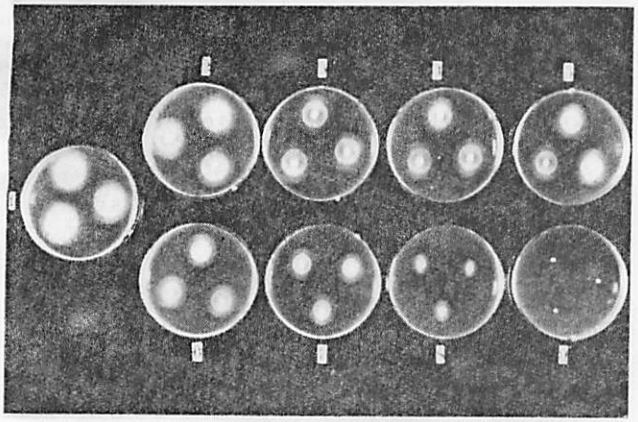

Fig. 2. Effect of a series of concentrations of α-methylene butyrolactone on the growth of *F. oxysporum* f. *tulipae* on potato dextrose agar. 1 = control (no lactone added); 2 = 12 ppm; 3 = 24 ppm; 4 = 36 ppm, etc.; 9 = 96 ppm lactone added, fungus killed.

lactone which could be extracted from a given weight of skin tissue was found to diminish rapidly during the last weeks before the skins turn brown. In extracts made from the brown skin it could not be found. Therefore it is not surprising that after inoculation of the brown skin with *F. oxysporum* in the way described by Bergman (1966), infection of the underlying undamaged fleshy scales occurred. The same lactone could be isolated from other parts of the tulip plant in considerable quantities, especially from the flower pistils, the upper part of the stem, the tips of the youngest leaves, the roots and the lowest, subsoil part of the stem. Schönbeck (1966, 1967), in his work on flower infection, found that macerated tissue of the tulip flower pistils and of several other parts of the plant was toxic to a number of fungi. His experimental results suggest that he has been dealing with the same substance.

In extracts made from the outer fleshy bulb scale the lactone was also found. Nearly all lactone isolated from this part of the bulb originated from the outermost few cell layers, as was demonstrated by separate extraction of different layers parallel to the surface. A few weeks before harvest the extract of the outermost layer of the scale tissue contained as much of the fungitoxic principle as an extract made from the skin. In the extracts made from the outermost layer of the scale tissue the concentration of the fungitoxic substance decreased considerably during the last few weeks before harvest, but increased again a few weeks after the bulbs had been stored.

This is in agreement with the results of a series of weekly inoculations of the undamaged outer fleshy scale. These inoculations were performed after removing the white (respectively, the brown) skin whereby care was taken to avoid wounding of the underlying scale. A small piece of tissue cut from a *Fusarium*-diseased bulb was placed on the undamaged outer scales and covered with a piece of moist filter paper. The bulbs were buried in moist sand and placed at 28 °C for 10 days. When inoculations were performed until a few weeks before harvest time never more than 60 % of the bulbs became diseased. However, when inoculation took place during the last weeks before and the first weeks after harvest, the percentage of successful inoculations

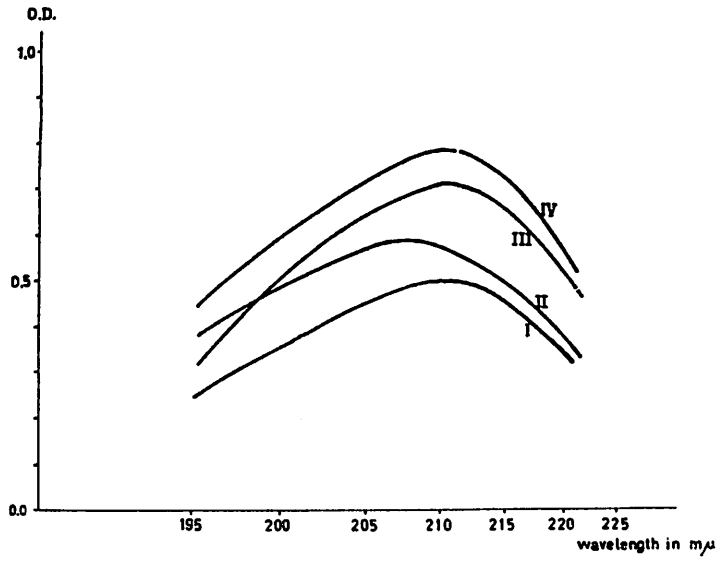

Fig. 3. Ultraviolet spectra of solutions of α-methylene butyrolactone and its precursor. I: 6.5 × 10^{-5} M α-methylene butyrolactone in aqua bidest; II: precursor in aqua bidest; III: precursor in Sørensen phosphate buffer 0.005 M pH 7.5; IV: as II, but after heating at 80 °C for 15 min. I, III and IV: maximal absorption at 211 mμ, II at 206–207 mμ.

gradually increased to nearly 100%; at later inoculations, however, this percentage decreased again.

It must be emphasized that a superficial wounding of the fleshy scale (e.g. by rubbing with fine carborundum powder) always resulted in heavy infections.

Evidence for the existence of a precursor

There are indications that the fungitoxic lactone is not present as such in the tulip plant tissue. Upon grinding in acetone or in acid buffer (pH lower than 6.0), only small amounts of the lactone could be found in the extract. However, when such an extract was mixed with potato dextrose agar together with buffer of pH 7.5, growth of *F. oxysporum* was inhibited as with the pure lactone. Thus, it seems probable that a precursor is present, which itself is not fungitoxic and which is unstable at pH's above 6.0. Independently the same lactone has been extracted from tulip bulb scales by Brongersma-Oosterhoff (1967), who supposed the precursor to be the glycoside of this compound. The existence of such a glycoside had already been proposed by Cavallito and Haskell in 1946, who found the same lactone in extracts made from *Erythronium americanum*, another species of the Liliaceae.

From different parts of the tulip plant a substance, which might be a precursor, has been isolated and purified (Beijersbergen[1]). This substance immediately yielded the lactone in vitro in phosphate or Tris buffer pH 7.5, but in aqueous solution only after a 15 min treatment at 80 °C, as could be demonstrated by UV spectrophotometry

[1] To be published as part of a Ph. D. Thesis.

(Fig. 3). The precursor under discussion absorbs in the UV with a maximum at 206–207 mμ (II). At pH 7.5, the maximum shifts to 211 mμ (III). After heating a solution of the precursor at 80 °C, it showed an absorption spectrum as represented by curve IV. Curves III and IV are identical with the absorption curve of pure lactone (I).
In the bioassay it was found that the compound under discussion is not toxic to *F. oxysporum*, not even in high concentrations. After dissolving in buffer pH 7.5 or heating it became fungitoxic.
The identification of the possible precursor has not yet been accomplished, but glucose or a compound related to glucose was found to be part of the molecule.

Discussion

It is reasonable to assume that there is a causal relationship between the high concentration of lactone found in extracts made from the white skin, the roots and the stem of tulips and the fact that these parts are not attacked by *F. oxysporum* under circumstances normally prevailing in bulb cultivation in this country.
Obviously, the skin serves as a protective layer against infection of the underlying fleshy scales by *F. oxysporum* during the greater part of the growth period. Only when the concentration of the compound(s) directly or indirectly responsible for the protection diminishes, the fungus is able to reach the outer fleshy scale. However, infection of this part is possible only if the parasite is able to pass the second chemical barrier located in the outermost cell layers of the outer bulb scale. It is not known as yet, how the pathogen is capable to overcome this second barrier. Neither is it known whether the substance(s) under discussion are factors of main importance in the host–parasite relationship concerned. Therefore, at the present stage of our investigations it seems premature to suppose that, for instance, differences in susceptibility to *F. oxysporum* in various tulip varieties are related directly to the presence of the lactone or its precursor in the plant tissue.

Samenvatting

Isolatie van een fungitoxische stof uit tulpen en de mogelijke betekenis daarvan bij de bescherming tegen ziekten

Een belangrijke ziekte in de tulpencultuur wordt veroorzaakt door een gespecialiseerde vorm van *Fusarium oxysporum*. Uit veldwaarnemingen werd geconcludeerd dat de schimmel gedurende een groot gedeelte van het groeiseizoen niet in de tulpebol kan binnendringen. De witte huid bleek een barrière tegen het binnendringen van de schimmel te vormen. Uit extracten van de witte huid (en van andere delen van de tulpeplant) werd een fungitoxische stof geïsoleerd, die geïdentificeerd is als α-methyleen butyrolacton. Een verbinding werd geïsoleerd, die onder bepaalde omstandigheden in vitro in het lacton overgaat en mogelijk als precursor fungeert. De implicaties van de aanwezigheid van de fungitoxische stof in de verschillende delen van de plant voor de vatbaarheid voor *Fusarium* worden besproken.

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