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PRESENCE OF A SUBSTANCE IN THE WHITE SKIN OF YOUNG TULIP BULBS WHICH INHIBITS GROWTH OF *FUSARIUM OXYSPORUM*[1]

De aanwezigheid van een stof in de witte huiden van jonge tulpebollen, welke de groei van Fusarium oxysporum *remt*

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In field infections of tulip bulbs caused by *F. oxysporum*, soil temperature, although important, has been proved to be not the decisive factor influencing the moment of infection. The presence in the white skin tissue of young bulbs of a water soluble substance which has a growth inhibiting effect on *F. oxysporum in vitro* has been demonstrated. Inoculations with the fungus on the white skins were unsuccessful. In the susceptible tulip cultivar so far examined the concentration of this substance in extracts decreases during the last weeks before the bulbs mature. It is suggested that this substance is protecting the susceptible fleshy bulb scales during the greater part of the growth period and that its diminution during the final weeks enables *F. oxysporum* to infect the scales during this period.

INTRODUCTION

In a previous paper (BERGMAN, 1965) it was stated that field infection of tulip bulbs by *Fusarium oxysporum* Schlecht. mainly takes place directly into the fleshy outer scales of the young bulbs and usually during the last weeks before harvest. It was suggested that this phenomenon could be ascribed at least partly to the soil temperature, which is usually highest during the first weeks of July, for it has been proved that high soil temperature favours field infection by *F. oxysporum*.

The present paper deals with a factor of a chemical nature present in the bulb skin which may inhibit the infection.

Even when a considerable amount of inoculum is present at the time of planting – either in or on the planted bulbs, or in the soil – the tulip plant and the young bulbs develop in a normal way. The presence at the time of harvesting of *F. oxysporum* infections too small to be recognized suggests that: 1. the pathogen is only able to penetrate during the last weeks before harvest, or 2. infection has occurred earlier but the fungus has not been able to develop in the tissues of the young bulb.

This could be explained by assuming: either that the temperature is too low for fungal growth during the greater part of the growing season, or that the scales of the young bulb are an unsuitable substratum for the development of *F. oxysporum*.

These possibilities have been investigated in the following experiments.

INFLUENCE OF HIGH SOIL TEMPERATURE DURING DIFFERENT PERIODS OF PLANT GROWTH ON INFECTION IN THE YOUNG BULB BY *F. OXYSPORUM*

In October 1964, healthy bulbs of the cultivar 'Red Giant', susceptible to

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F. oxysporum, were inoculated by dipping in a spore suspension immediately before planting in metal containers filled with dune sand. The containers were kept at a temperature of 5 °C to induce root and shoot development, until in March they were transferred to Wisconsin temperature tanks kept at 6 °C (later 10 °C) soil temperature.

Starting 20 April (when the plants just began flowering) two containers, each with eight plants, were transferred every week to a soil temperature tank at 22 °C. After two weeks' growth at this temperature the plants were dug up and the young bulbs cleaned and disinfected in an organic mercury compound for five minutes. It has been proved (unpublished experiments) that mycelium and microconidia of *F. oxysporum* of tulips adhering to the outside of a bulb are killed by this disinfection method. The bulbs were then cut in slices about 3 mm thick and these slices were plated on potato dextrose agar with antibiotics added (BERGMAN, 1965). After incubation the number of bulbs from which *F. oxysporum* had grown out on the agar was counted. The results are summarized in Table 1.

TABLE 1. Number of young tulip bulbs showing growth of *F. oxysporum* after outside disinfection, cutting in slices and plating on potato dextrose agar. Lots of 16 plants, lifted after two weeks' exposure to 22 °C soil temperature during different stages of plant development; previous to this period soil temperature was held at 6–10 °C.

Aantal bollen, waaruit na uitwendige ontsmetting, snijden in plakken en uitleggen op aardappelglucose-agar F. oxysporum *groeide. Groepen van 16 planten, die werden opgegraven na gedurende twee weken bij 22 °C bodemtemperatuur gestaan te hebben tijdens verschillende stadia van de groei; voordien bij 6–10 °C.*

Period at 22 °C soil temperature	Number of bulbs with *Fusarium*	Period at 22 °C soil temperature	Number of bulbs with *Fusarium*
20/4– 4/5	0	1/6–15/6	3
28/4–12/5	0	8/6–22/6	3
4/5–18/5	0	15/6–29/6	2
11/5–25/5	0	22/6– 6/7	6
18/5– 1/6	0	6/7–20/7	8[1]
25/5– 8/6	1	13/7–27/7	7[1]
Periode bij 22 °C bodemtemperatuur	*Aantal bollen met* Fusarium	*Periode bij 22 °C bodemtemperatuur*	*Aantal bollen met* Fusarium

[1] Owing to the preceding low soil temperature the plants were still completely green at 6/7 and 13/7, but when harvested they were dying and the young bulb skins were yellowing and membranous.

Ten gevolge van de voorafgaande lage bodemtemperatuur waren de planten op 6/7 en 13/7 nog geheel groen, maar bij de oogst vrijwel afgestorven en de huiden van de jonge bollen vergelend en vliezig.

Though all lots had been grown at a soil temperature favourable for *F. oxysporum* infection for two weeks prior to digging, only the nearly full-grown bulbs in the lots exposed to this temperature during the later stages of growth proved to be appreciably infected. The young and still small bulbs in the firot five lots escaped infection completely, in spite of the considerable amount of inoculum added. Repetitions of this experiment in a similar way gave the same results. This demonstrates that soil temperature is not the only factor influencing infection in the soil.

SUITABILITY OF SCALE TISSUE OF YOUNG BULBS AS A NUTRIENT MEDIUM FOR GROWTH OF *F. OXYSPORUM*

From a stock of tulips cv. 'Red Giant' grown under natural conditions a number of plants was lifted at weekly intervals. On each occasion fifty bulbs were cleaned and the bulb skin (which was white and fleshy at first but gradually became pigmented and membranous in the first weeks of July) was removed. The exposed outer fleshy scales were wounded with fine carborundum in a standardized way and a drop of water containing about 1000 microconidia of *F. oxysporum* was applied to the wound. The inoculated bulbs were incubated at 25°C and 100% relative humidity.

After eight days the number of bulbs was counted on which a diseased spot of more than 7 mm diameter had developed. This dimension gave the certainty that the inoculation had resulted in a rapid development of the fungus in the host tissue.

TABLE 2. Number of bulbs showing diseased areas more than 7 mm in diameter in the outer scale, caused by *F. oxysporum*, eight days after superficial wounding and inoculation with a conidial suspension; inoculations at intervals during the growing period and after harvest; lots of 50 bulbs.

Aantal bollen met door F. oxysporum *veroorzaakte plekken van meer dan 7 mm diameter, acht dagen na inoculatie met een sporensuspensie op een oppervlakkige verwonding van de buitenste rok op verschillende tijdstippen tijdens het groeiseizoen en na de oogst; 50 bollen per rooidatum.*

Inoculated on	Number of bulbs	Inoculated on	Number of bulbs
17/5	34	7/7	25
24/5	39	14/7	46
31/5	45	21/7[1]	46
7/6	38	28/7	44
14/6	45	11/8	31
Inoculatiedatum	*Aantal bollen*	*Inoculatiedatum*	*Aantal bollen*

[1] Plants dying, remaining bulbs harvested 22/7 and stored.
Planten afgestorven, resterende bollen op 22/7 geoogst en opgeslagen.

The data of Table 2 show that even the very young bulbs (two months before normal harvesting time, when they are still very small) are a substratum very suitable for *F. oxysporum*. Therefore, this result does not explain the fact demonstrated in the preceding experiment that tulip bulbs are usually not invaded until the last weeks before the bulbs mature. It seemed probable that a barrier of some kind might account for this situation, this barrier being present in the tissues surrounding the young bulbs (either in the white skin or in the decaying scales of the old bulb).

DEMONSTRATION OF THE PRESENCE OF A BARRIER IN THE WHITE BULB SKIN PREVENTING INFECTION OF THE BULB SCALES BY *F. OXYSPORUM*

On 17 June, that is about five weeks before the bulbs matured, a hundred bulbs from the same stock of cv. 'Red Giant' were lifted and cleaned. From half of this number the skins were removed, these being still white and fleshy and

nearly as thick as the scales at that moment, and the exposed bulb scales were inoculated with a spore suspension of *F. oxysporum* in the way described. The other half was inoculated on the white skins. All bulbs were then incubated at 25 °C and 100 % relative humidity. After eight days all bulbs inoculated on the outer scales were heavily infected, while those inoculated on the white skins (which turned yellowish-brown during the incubation period) were undamaged. The fungus had not spread into the inoculated skin (Fig. 1) and the underlying bulb scale had not been invaded (Fig. 2). The experiment was repeated several times with other cultivars and in later stages of development of the bulbs, when the skins had turned more membranous but were still white. Inoculations on the white skins were never successful, these results supporting the supposition already mentioned about the existence of a barrier in the white skin.

EXTRACTION OF AN ACTIVE PRINCIPLE FROM WHITE TULIP SKINS

The following extraction procedure was applied. Samples of twenty five grammes (fresh weight) of white skins of the susceptible variety 'Red Giant' were collected about one month before normal harvesting time. The samples were ground with 50 ml of a phosphate buffer solution of 1/15 M (pH 7.5) in a Servall omni-mixer at high speed (15000 r.p.m.) for one minute. The fluid was separated from the pulp through a Buchner filter.

In the same way extracts were made from the old decaying scales of the mother bulbs, which surrounded the young bulbs.

In order to inactivate enzymes, which might have a disturbing influence, half of the extracted fluids were heated at 80 °C for half an hour. All samples were

TABLE 3. Diameters of colonies of *F. oxysporum* after two days' incubation on 10 ml p.d.a. mixed with 3 or 5 ml of extracts made from decaying scales of old bulbs or from the white skins of young bulbs; means of three replicates.
Diameters van kolonies van F. oxysporum, *na twee dagen incubatie gegroeid op 10 ml a.g.a. vermengd met 3 of 5 ml extract uit oude rokken van de moederbol of uit de witte huid van jonge bollen; gemiddelden van drie herhalingen.*

Kind of tissue	Extract heated ½ hour at 80 °C	Diameter of *Fusarium* colonies (mm) Extract per dish	
		3 ml	5 ml
1. Scales of old bulb *Oude bolrokken*	no *niet*	29.3	29.3
2. as 1. *als 1.*	yes *wel*	27.3	25.2
3. White skin of young bulb *Witte huid van jonge bol*	no *niet*	0	0
4. as 3. *als 3.*	yes *wel*	0	0
5. Control (5 ml phosphate buffer only) *Blanco (alleen 5 cm³ fosfaat-buffer)*		28.9	
Soort weefsel	*Extract verwarmd ½ uur bij 80 °C*	*Diameter van de* Fusarium-*kolonies (mm) Extract per schaal*	

centrifuged at 3000 r.p.m. for 15 minutes and the clear fluids were sterilized by passing through a Seitz bacterial filter.

Amounts of 3 and 5 ml of these sterile extracts were mixed in petri dishes with 10 ml potato dextrose agar (p.d.a.). On each dish three pieces of 3 mm diameter of a p.d.a. culture of *F. oxysporum* from tulip were plated. After two days' incubation at 25 °C the diameters were measured of the *Fusarium* colonies grown from these inoculations (Table 3).

These data establish the existence of a water soluble substance in the white skins of young tulip bulbs which inhibits growth of *F. oxysporum* in an artificial medium containing an appropriate amount of this extract. The presence of a similar active principle could not be demonstrated in the decaying scales of the mother bulb.

CONCENTRATION OF THE ACTIVE SUBSTANCE IN EXTRACTS OF WHITE BULB SKINS AT DIFFERENT STAGES OF BULB DEVELOPMENT

From a stock of cv. 'Red Giant' grown in the garden, samples were taken on 23 June, 5, 13 and 19 July. The remaining bulbs of this stock were harvested on 22 July, when the bulbs were mature and the skins had turned brown and membranous. The bulb skins of the four samples were frozen at -30 °C. At the end of July all were extracted as described. The sterile extracts were pipetted in the dilution range from 0.5 to 2.5 ml per dish. The volumes were made up to 5 ml with sterile buffer solution, mixed with 10 ml potato dextrose agar and the plates were inoculated with pieces of *Fusarium* agar as described.

The results, given in Fig. 3, show that 1½ ml of extract per dish from the skins sampled on 23/6 and 5/7 caused complete or nearly complete inhibition of growth of *F. oxysporum*. With extract of skins sampled on 13/7 a similar result was obtained only when 2 ml per dish had been added. The extract of skins collected on 19/7 (three days before maturity of the bulbs) did not inhibit growth, even at 2½ ml extract per dish.

It is not yet possible to estimate the real content of active substance in the white skin, because fresh weight samples were used and because it is not yet known what proportion of the content of active principle is isolated with the extraction method used. However, the results given in Fig. 3 demonstrate that the concentration of extractable active substance in the skins decreases rapidly when the bulbs of this cultivar mature.

It proved to be impossible to extract any active substance from the brown and membranous skins of mature bulbs, even when the skins – after sterilisation in propylene oxyde for two days – had been soaked for several days in the sterile buffer solution prior to grinding.

DISCUSSION

Since the work of WALKER and co-workers on the resistance of onions to smudge, *Colletotrichum circinans* (Berk.) Vogl. (WALKER, 1923; LINK, ANGELL & WALKER, 1929; LINK & WALKER, 1933; and other papers) much attention has been paid to pre-existing toxins in relation to disease resistance in plants. In the reviews on this subject given by various authors there is much diversity of opinion about the extent to which these investigations have provided conclusive evidence.

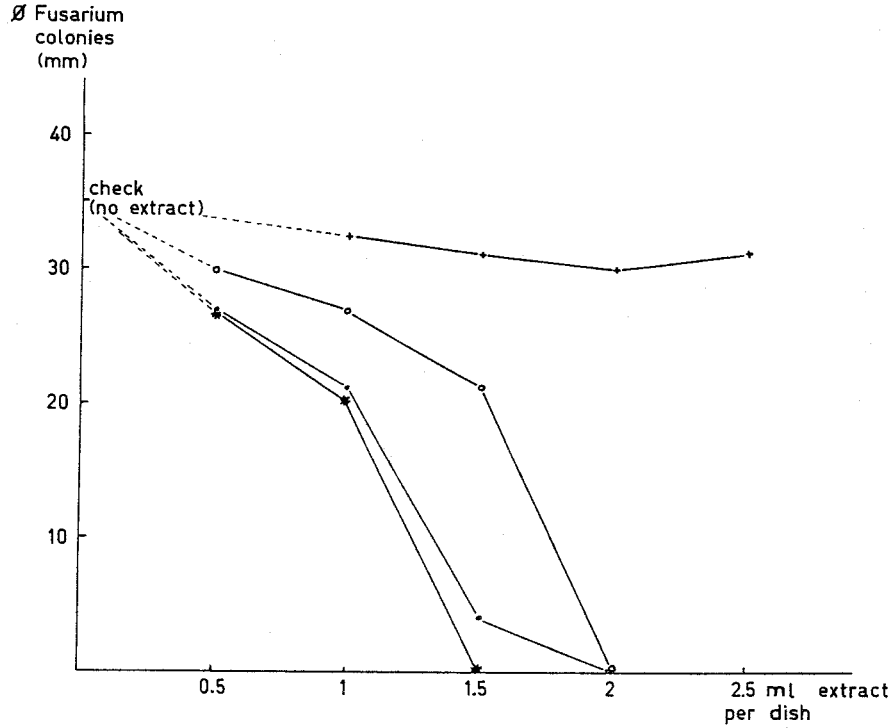

FIG. 3. Growth of *F. oxysporum* (mm) after four days' incubation at 25°C on potato dextrose agar mixed with different quantities of sterile aqueous extracts made from white tulip bulb skins cv. 'Red Giant', which were sampled at four times during the last weeks of bulb growth (· — · : extract from skins sampled on 23 June, * — * : do on 5 July, o — o : do on 13 July, + — + : do on 19 July).
Remaining bulbs mature and harvested on 22 July.

Groei van F. oxysporum *(in mm) na vier dagen incubatie bij 25 °C op aardappel-glucose-agar, gemengd met verschillende hoeveelheden steriel waterig extract van witte tulpehuiden cv. 'Red Giant', welke op vier tijdstippen tijdens de laatste weken van de groeiperiode werden verzameld (· — · : extract van huiden verzameld op 23/6, * — * : idem op 5/7, o — o : idem op 13/7, + — + : idem op 19/7).*
Resterende bollen rijp geoogst op 22/7.

In his short review on toxins of this nature CRUICKSHANK (1963) considers the investigations on onion smudge to be the only work so far supported by sufficient proof.

FARKAS & KIRÁLY (1962) are of the opinion that in a number of cases a parallelism has been found between phenol content and disease resistance of plant varieties, especially when these substances were localized in non-living tissues. When the possibility was investigated of a correlation between phenol content in actively metabolizing organs and disease resistance, the results in their opinion were less conclusive.

WALKER & STAHMANN (1955) emphasize that whether or not an organism becomes a pathogen and the degree of pathogenicity it develops is determined largely during the interaction of host and parasite. In this respect these authors

regard the situation with onion smudge as unusual, because penetration of the parasite is prevented by a resistance character in the dead tissue at the portal of entry and depends upon the fact that the chemicals involved are water soluble.

ALLEN (1959) distinguishes between diffusable fungitoxins from dead tissues of which he gives several examples and toxic substances present in the living cells of resistant plants. In the latter category it is, according to ALLEN, not sufficient to establish that a resistant plant yields a substance toxic to the pathogen nor even to show a correlation between resistance and yield of toxic principle. If the substance inhibits virulent and avirulent strains alike, or if the substance is not found at the portal of infection or in concentrations required to inhibit, the evidence for its being responsible for resistance is hardly convincing. WALKER & STAHMANN (l.e.) express the same opinion and give examples of toxic substances present in plant tissue cells which do not inhibit infection. The most striking case is the presence of several toxic compounds in the epidermal cells of the fleshy scales of colored onion bulbs. Because these substances are confined to the living cell and are destroyed by fungus exudates before they come in contact with the hyphae, they do not inhibit infection by *Colletotrichum circinans.*

Reviewing the results of the experiments given in this article the following points are of major interest:

1. most infections of tulip bulbs by *F. oxysporum* occur during the last weeks before the bulbs mature;
2. soil temperature, though important in relation to infection, is not a decisive factor;
3. the fleshy bulb scales are a very suitable substratum for the fungus;
4. in an aqueous extract made from the white skins of young bulbs a substance is present which has a strong growth inhibiting effect on *F. oxysporum in vitro;* inoculations with the fungus on the slightly wounded white skin are unsuccessful;
5. in the susceptible cultivar so far examined the concentration of extractable active principle decreases during the last weeks of growth.

From these facts it is very plausible to assume that the active principle in the skin forms a barrier which inhibits infection by *F. oxysporum* during the greater part of the growth period. Only during the last weeks of growth does the concentration of this substance decrease to such an extent that infection – perhaps further favoured by higher soil temperature – can take place in the scales, because the skin no longer forms a barrier.

ALLEN (1959) formulated KOCH's postulates in such a way that they could be applied to the experimental proof of the role of a chemical substance in disease resistance. The first two postulates ("association of the substance with the protection, at the site where protection occurs", and "isolation of the inhibitory agent") render no special technical problems. Since it has been proved (BERGMAN, 1965) that most field infections take place directly through the skin into the fleshy scales, the last part of the first requirement has been fulfilled, as is the second postulate. Because there seems to be a causal relationship between the normal period of infection and the fact that just in this period the demonstrable concentration of the active substance decreases, it may be assumed that the first requirement is also met. However, in the writer's opinion it is hardly pos-

sible to conform to ALLEN's next two postulates: "introduction of the substance to the appropriate loci conferring protection" and "resemblance of the nature of the protection thus induced to that of the natural agent".

It is not known whether the loss of active substance is due to a transposition into an insoluble or otherwise inactive form. It is also possible that the substance is translocated to other parts of the bulb, together with other constituents of the white skin. SCHÖNBECK (1966) found a substance toxic to *Pythium debaryanum* Hesse and several other fungi in the pistils of tulip flowers and in a lower concentration in the lower parts of the plant. It is not yet known whether this toxin is identical with the substance dealt with in this paper.

The pigments giving the skin its ultimate brown colour are probably not directly related to the active substance. When the extract of the white skins is heated at 80 °C for half an hour the fluid is stained deep brown but the activity is not lost to any extent. This points to a situation comparable with that of the pigments present in the dry outer scales of colored onions (flavones and anthocyanins) which do not inhibit growth of *Colletotrichum circinans* (ANGELL *et al.*, 1930).

On the other hand the situation in tulips differs from that in onions insofar that in the latter the toxic substances (protocatechuic acid and catechol) are released from the scales only when the scale tissue has died. In tulips the highest concentration in extracts is found when the skin tissue is living, whereas it diminishes when the skin turns membranous and cannot be demonstrated in the brown and dead skin tissue.

However, when considered strictly, the experiments described do not establish that the substance under consideration is active *in situ* in the living cell. Activity demonstrated when inoculations were made on the skin wounded by carborundum might also be attributed to the liberation of the substance from the damaged cells. However, there are indications that without wounding the substance in the white skin has also a protective effect against infection by *F. oxysporum*.

The mechanism of protection is still obscure, but free diffusion of the substance seems improbable.

Further work on biological activity, physical properties and purification are in progress and will be published before long in this journal. For practical purposes the name "tulipalin" is given to the substance under consideration.

SAMENVATTING

Het is gebleken, dat de bodemtemperatuur – hoewel van invloed – niet de beslissende factor is bij de infectie van tulpebollen in het veld door *Fusarium oxysporum*. Wanneer de bodemtemperatuur vroeg in het groeiseizoen tot een voor infectie gunstige hoogte werd opgevoerd, vond geen of nauwelijks infectie van de jonge bollen plaats. Dit werd pas in enige omvang het geval gedurende de laatste weken van het groeiseizoen (tabel 1). Dit resultaat kan niet geweten worden aan de samenstelling van de vlezige jonge bolrokken, want deze vormen zeer vroeg tijdens de ontwikkeling een goed substraat voor *F. oxysporum* (tabel 2). Wanneer echter geïnoculeerd werd op de witte huid van jonge bollen, vond geen infectie plaats (fig. 1, 2). Uit de witte huiden kon een waterig extract bereid worden, dat na menging met aardappel-glucose-agar de groei van *F. oxysporum* volkomen verhinderde (tabel 3). Bij een voor *F. oxysporum* gevoelige tulpecultivar bleek, dat het gehalte aan deze stof of stoffen in de extracten afnam naar-

mate de huiden korter vóór de normale rooidatum werden geëxtraheerd (fig. 3). Op grond van deze waarnemingen mag worden aangenomen, dat dit actieve principe in de huid de onderliggende rokken van de bol gedurende het grootste gedeelte van het groeiseizoen beschermt en dat deze beschermende werking pas kort vóór de oogst verdwijnt.

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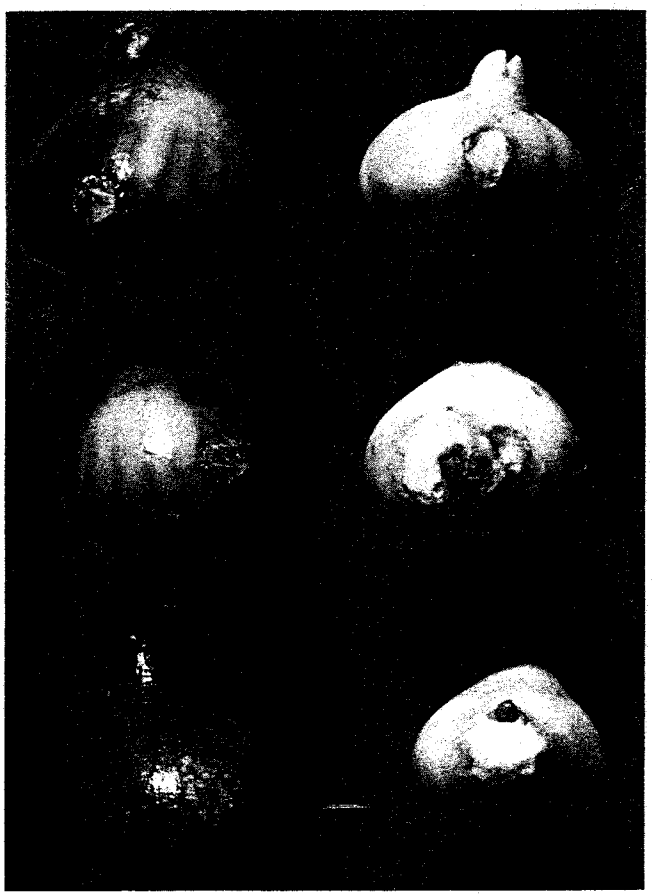

FIG. 1. Tulip bulbs cv. 'Red Giant' inoculated with a spore suspension of *F. oxysporum* on a carborundum-inflicted wound, after eight days incubation at 25 °C and 100% r. h. Left row: inoculation on the white and fleshy skin; no growth of *F. oxysporum* (the grey spots on the skins, which turned brown during incubation, are caused by the carborundum powder).
Right row: inoculation on the fleshy outer bulb scale; rapid growth of *F. oxysporum*.
Tulpebollen cv. 'Red Giant', geïnoculeerd met een sporensuspensie van F. oxysporum *op een verwonding met behulp van carborundum veroorzaakt, na acht dagen incubatie bij 25 °C en 100% relatieve luchtvochtigheid.*
Linker rij: inoculatie op de nog witte en vlezige huid, geen groei van F. oxysporum *(de grijze vlekken op de huiden, welke tijdens incubatie bruin werden, zijn veroorzaakt door het carborundumpoeder).*
Rechter rij: inoculatie op de buitenste bolrok, sterke groei van F. oxysporum.

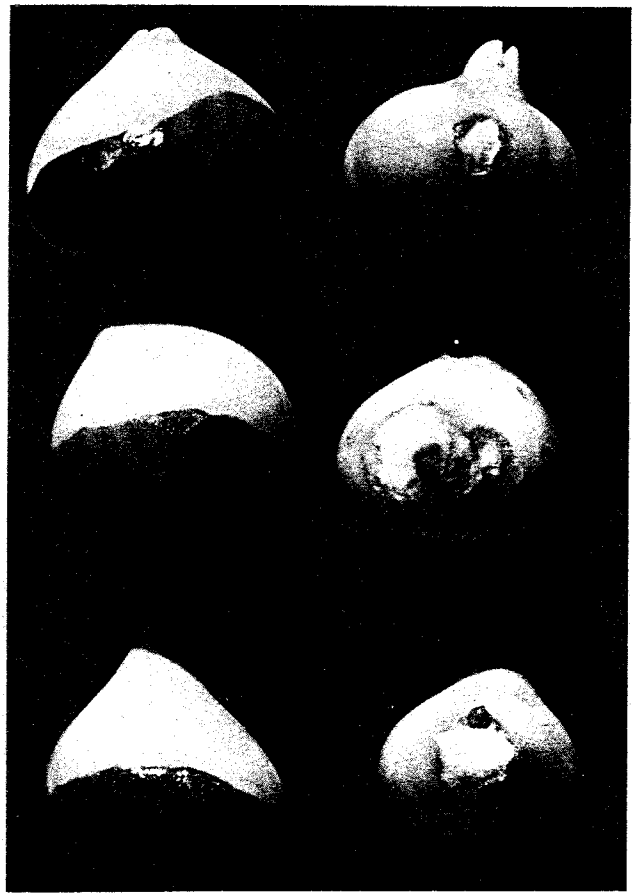

FIG. 2. Same bulbs as in Fig. 1. From the bulbs in left row the skins have been partly removed to show that the underlying scales are not affected by *F. oxysporum*.

Dezelfde bollen als in fig. 1. Van de bollen in de linker rij is de huid gedeeltelijk verwijderd om te tonen dat de onderliggende bolrok niet is aangetast door F. oxysporum.