Floret Development and Teliospore Production in Bunt-Infected Wheat, in Planta and in Cultured Spikelets

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Accepted for publication 18 May 1989 (submitted for electronic processing).

ABSTRACT

Trione, E. J., Stockwell, V. O., and Latham, C. J. 1989. Floret development and teliospore production in bunt-infected wheat, in planta and in cultured spikelets. Phytopathology 79: 999-1002.

The formation of teliospores in the common and dwarf bunt-infected ovaries began when the spikes were about 65–70 mm long and the ovaries were 0.5 mm in diameter. In infected florets, the anthers developed abnormally, viable pollen was not produced, the filaments elongated only slightly, and anthesis did not occur. Infected ovaries were not fertilized, but they enlarged at a faster rate and achieved a larger size than the healthy ovaries. Greening of the infected ovaries was correlated with the onset of teliospore formation in the ovary, and as sporulation continued, the greening intensified. Healthy spikelets usually supported only two developing grains (the distal florets aborted), but 6–10 infected kernels were commonly produced per spikelet on dwarf-bunted plants. The possible involvement of hormones in the abnormal morphological alterations of the infected florets is discussed. Excised wheat spikelets infected with *Tilletia controversa* were successfully grown in liquid culture media containing sucrose, glutamine, salts, and minor organic factors. Varying the composition of the medium affected sporulation in the ovaries, and the highest sporulation occurred at 3 g L^{-1} of glutamine.

Additional keyword: Tilletia caries.

Wheat bunts have been serious wheat diseases since antiquity (Theophrastus, 371–287 B.C.), although the scientific records began much later (Tillet, 1755), according to Fischer and Holton (2). Wheat plants infected with the bunt fungi undergo major morphological changes, especially in the florets. General field observations of bunt-infected florets have indicated that viable pollen does not develop, the ovaries of infected florets are larger than those in healthy florets, and there are more bunt-infected kernels per spikelet than seeds per spikelet on a healthy plant (7).

Excised wheat spikelets have been successfully grown, from anthesis to maturity, in an aseptic liquid culture medium containing sucrose, glutamine, salts, and minor organic factors (10). In these cultured spikelets, grain development paralleled in vivo development. This in vitro technique affords control over the nutrient supply to the spike and may be used to study the effects of many variables on healthy seed or infected seed development.

The purposes of this study were: 1) to quantify the morphological development of uninfected and bunt-infected wheat spikes and florets, and to compare the effects of common bunt, *Tilletia caries* DC. (Tul.), and dwarf bunt, *T. controversa* Kuhn, on anther and ovarian development; and 2) to determine the utility of spikelet culture in bunt research by measuring teliospore production in wheat ovaries in planta and in detached spikelets cultured at several sugar and glutamine levels.

MATERIALS AND METHODS

A dwarf bunt-susceptible winter wheat (*Triticum aestivum* L.), selection FW 77082 (PI 178201/2/WA × Hyslop; provided by M. Kolding, Hermiston, OR), was seeded in the field in October 1986 and inoculated with *T. controversa* by sprinkling a dense aqueous suspension of spores over the seeded rows and covering with vermiculite (7). The seedlings became established in the fall and were covered with snow for more than 60 days in the winter. Those conditions were ideal for dwarf bunt infection to occur. In the spring, when the plants were in the three- to four-leaf stage, they were transplanted to the greenhouse (22 ± 3 C, 16-hr photoperiod) for more uniform development, observation, and harvest. Susceptible wheat plants, cultivar Red Bobs, were also inoculated with spores of *T. controversa* and *T. caries* and maintained in the greenhouse as previously described (8). The lengths of ovaries and anthers in developing spikelets were measured to 0.1 mm with an eyepiece micrometer in a stereomicroscope, and analysis of variance was performed on the data.

In vitro culture of infected spikelets. Bunt-infected wheat spikes (3–3.5 cm long) in the boot stage, Feekes' stage 10 (5), were cut from greenhouse-grown plants. The surface of the leaf covering the spike was disinfected with sodium hypochlorite (0.05%) and rinsed three times with sterile water. The outer leaf was removed carefully, and the six central spikelets (including adjacent rachis tissue) were each excised from the spike and placed upright in the liquid medium (10) in wells of a cluster culture dish as previously described (10). Four replicate spikelets were used per treatment. The spikelet culture media (A-I) all contained the same salts and vitamins (10) but various amounts of sucrose (10, 30, and 90 g L^{-1}) and L-glutamine (1, 3, and 9 g L^{-1}). The culture dishes were incubated at 21 C (14 hr of fluorescent light at 2,500 mW m^{-2}) and 19 C night. At regular intervals spikelets were removed, the primary basal ovaries were excised, and the fresh weight and the number of teliospores in each bunt ball was determined.

Determination of the number of teliospores per bunt ball. The primary basal bunt ball was homogenized gently in distilled water (10 ml) containing one drop of Triton X-100. The homogenate was filtered through a 36-μm nylon mesh and the teliospores collected on a 5-μm filter disk. The spores were washed from the filter with deionized water (one drop of Triton X-100 per 10 ml) and centrifuged. The supernatant was removed by aspiration, and the spores were resuspended in 5 ml of 1% NaCl containing one drop of 6 mM ethylmercurithiosalicylate to prevent microbial growth.

The number of teliospores present in each sample was deter-

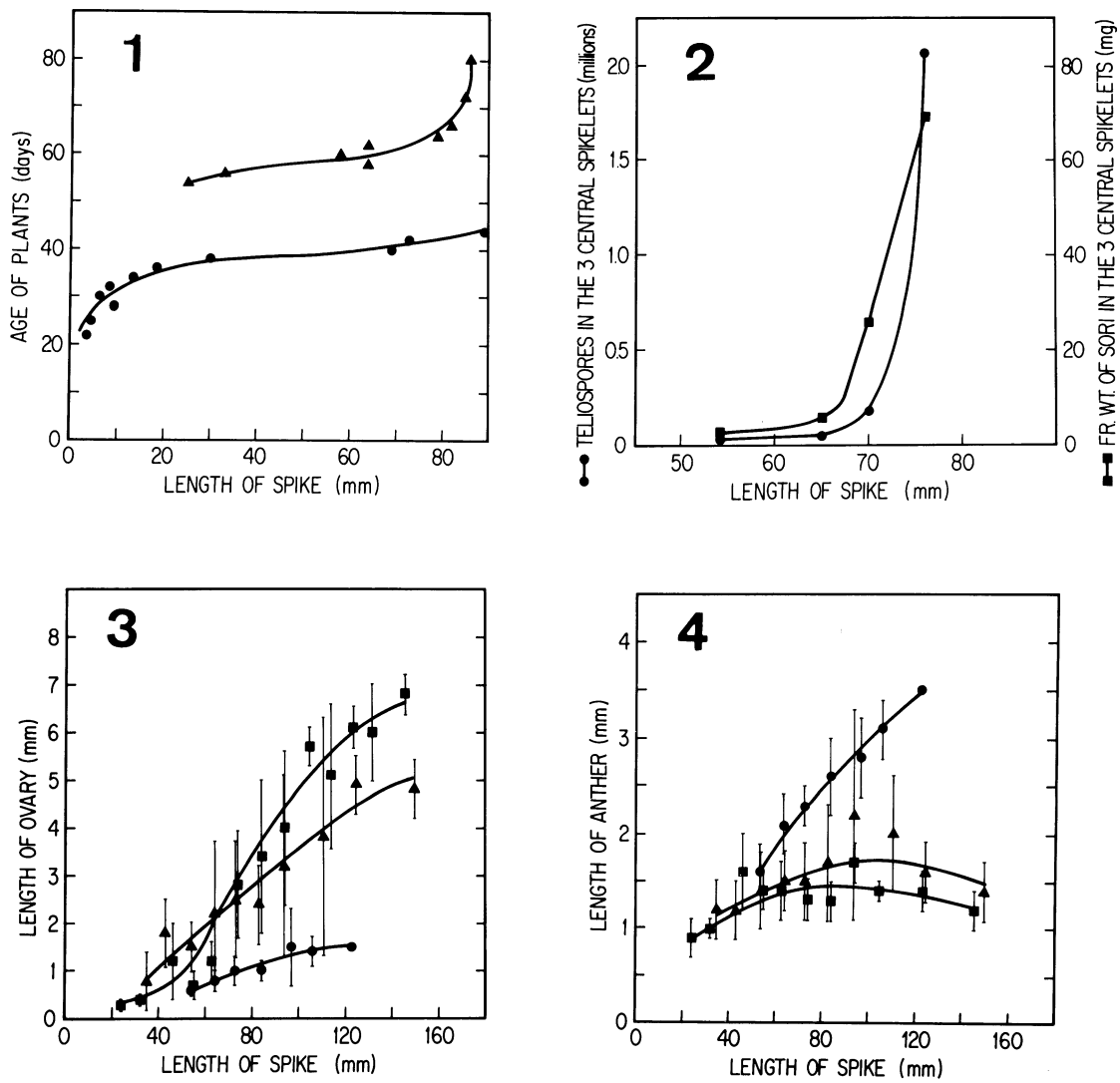
mined electronically with a flow cytometer (Coulter Counter, Model ZBI). Three counts were made of the control solution (containing all ingredients except spores), and three counts were taken of each spore sample. The true counts were calculated by subtracting the background average from the sample counts, and analysis of variance was performed on the data.

Cytological techniques. Excised wheat tissues were vacuum infiltrated with the fixative (2% glutaraldehyde, 3% acrolein, 0.2 M cacodylate buffer, pH 7.3) and fixed at 4 C for 6 hr. After fixation, tissues were washed well with cacodylate buffer and then dehydrated through a graded ethanol series. Tissues were then embedded with Historesin, a glycol methacrylate compound. Sections (3 μm) were cut with a rotary microtome with a steel knife, dried onto glass slides, and stained sequentially with Gill's triple-strength hematoxylin, followed by Lee's methylene blue-basic fuchsin (3). The sections were mounted with Permamount.

RESULTS

Morphological development of healthy and infected wheat spikes and florets. The culms and spikes of healthy wheat plants began to elongate 2 wk earlier than the culms and spikes of plants infected with dwarf bunt (Fig. 1). About 1 wk after the infected spikes began rapid elongation, teliospore formation started in the infected basal ovaries of the central spikelets. Emergence of the spikes from the flag leaves occurred in both healthy and infected plants when the spikes were about 80 mm long. Although the infected spikes began to elongate rapidly after they reached a length of 10–20 mm, the rapid growth of the ovaries and the formation of large numbers of spores in the ovaries did not begin until the bunt-infected spikes were 65–70 mm long (Fig. 2).

In the field and greenhouse, wheat plants infected with common bunt, *T. caries*, showed similar but less extreme changes in dwarfing, tillering, and sori per spikelet than those described for dwarf bunt, *T. controversa*. In healthy or bunt-infected wheat plants, when the spikes were about 4–5 mm long, the ovaries and the anthers were each about 0.1 mm long. Thereafter, significant morphological differences appeared in healthy vs. bunt-infected florets. Cytological studies of common and dwarf bunt-infected ovaries revealed that teliospore formation began when the ovaries were about 0.5 mm in length. Thereafter, the periderm of infected ovaries began to turn green and became darker green as the kernels enlarged. The quantitative changes in the ovaries and anthers of common and dwarf bunted plants were very similar



Figs. 1–4. 1, Change in spike length related to age of wheat plants, cultivar Red Bobs, after sowing in the greenhouse. -●-, uninfected; $n = 6$; LSD = 8.2; -▲-, infected with *Tilletia controversa*; $n = 6$; LSD = 19.6. **2,** A comparision of the length of the spike with the total fresh weight of the basal sori (in the three central spikelets) and the total number of teliospores in those sori. The data are from four typical wheat plants, selection 77082, infected with *T. controversa*. **3,** A comparison of the length of spikes with the length of ovaries present in the primary floret of the central spikelet in wheat plants, cultivar Red Bobs, grown in the greenhouse. -●-, uninfected; -■-, infected with *T. caries*, ▲- infected with *T. controversa*; mean values ± SD based on $n = 5$. **4,** A comparison of the length of spikes with the length of anthers from the primary floret of the central spikelet in wheat plants, cultivar Red Bobs, grown in the greenhouse. -●-, uninfected; -■-, infected with *T. caries*; -▲-, infected with *T. controversa*; mean values ± SD based on $n = 5$.

(Figs. 3 and 4). The infected ovaries increased in length at a much faster rate than healthy ovaries, and they were three to four times as long as the healthy ovaries at anthesis (Fig. 3). The anthers that developed in infected florets initially had the same shape and size as anthers in uninfected florets; however, healthy anthers grew faster than those in infected florets following the onset of teliosporogenesis (Fig. 4). The anthers in infected florets reached a maximum length of 1.5–1.8 mm, in spikes that were 80–100 mm long (Fig. 4), but as the spikes elongated, the anthers became shorter and developed abnormal shapes (Fig. 5). In contrast, the healthy anthers elongated at a nearly linear rate, reaching a length of about 3.5 mm at anthesis (Fig. 4).

The healthy lodicules became swollen just before anthesis, pushing the lemma and palea apart. As the healthy florets opened, the filaments elongated and the pollen was shed. In the dwarf bunt-infected florets, which were chronologically near anthesis, the lodicules were dry and thin and did not swell to force the florets to open; furthermore, the filaments in the diseased florets showed only about 25% of the normal elongation (Fig. 5). Mature viable pollen did not develop in bunt-infected florets; the pollen cytoplasm was either absent or plasmolyzed, and the pollen wall collapsed.

Since bunt-infected florets did not open, and viable pollen was not produced, fertilization of the infected ovaries apparently did not occur. Normally, unfertilized healthy ovaries abort; however, the unfertilized ovaries of infected plants developed into seedlike organs, i.e., sori. Commonly, a plant infected with dwarf bunt produced eight to 10 infected kernels per spikelet (Fig. 5) compared with only two to three kernels on a healthy spikelet.

Teliospore production in wheat ovaries in vitro and in vivo. The rate of production of teliospores in *T. controversa* infected plants was studied by measuring spore production in the basal ovaries of the central spikelet in plants growing in the greenhouse (Fig. 2) and in detached spikelets cultured in synthetic media (Figs. 6 and 7).

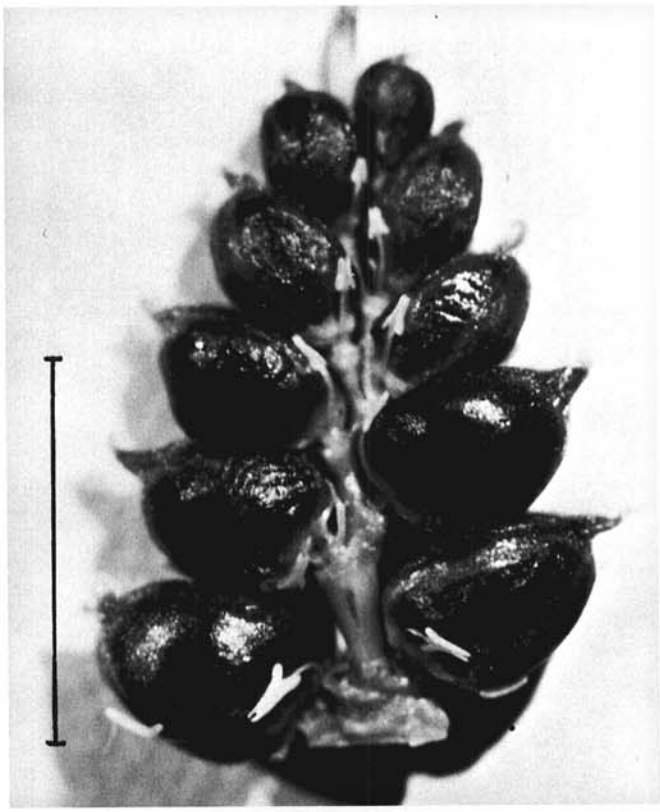


Fig. 5. Wheat spikelet, selection 77082, infected with *Tilletia controversa*. Anthers were abnormal and did not produce pollen, but ovaries developed into seedlike structures. Glumes, lemmas, and palea were removed. Bar = 1 cm.

In the detached spikelets infected with dwarf bunt, there was initially a mean of 1,105 teliospores per basal ovary with no significant differences between samples. After 16 days of incubation in the nine media containing various levels of sucrose and glutamine, teliospore production increased dramatically (Fig. 7) as determined by flow cytometry. The smallest spore production per ovary occurred in medium C, which was a 25-fold increase over the initial level, whereas the largest sporulation occurred in medium D with an 85-fold increase. Fisher's protected LSD test at 0.05 revealed that sucrose levels did not significantly affect teliospores per sorus, but glutamine at 3 g L^{-1} significantly increased spore production above glutamine at 1 or 9 g L^{-1}. Sucrose or glutamine alone did not significantly affect sorus weight, but the interaction of sucrose and glutamine was responsible for significant differences (Fig. 7). At extreme values, both sucrose and glutamine levels had a significant effect on sporulation per milligram fresh weight of sorus.

Spore production in the ovaries grown in vitro was much lower than in comparable ovaries in vivo. In intact plants the teliospore production was initially slow, but within 1 wk of the onset of sporulation it was increasing at an extremely rapid rate (Fig. 2). In the intact plant, 219,000 spores were produced per basal ovary in 7 days, compared with an increase to only 85,000 spores

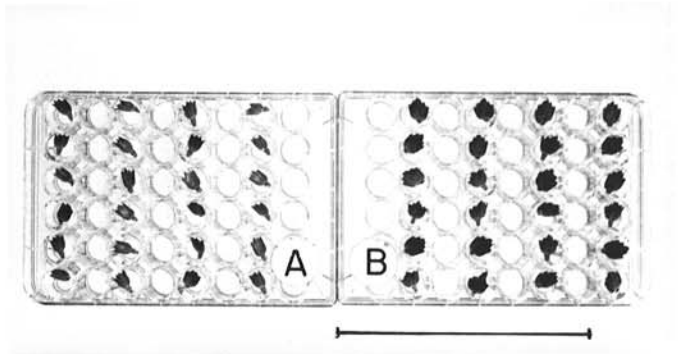


Fig. 6. Detached wheat spikelets, selection 77082, infected with *Tilletia controversa* growing in a liquid medium in a 48-well cluster culture dish. **A,** Wheat spikelets at beginning of the experiment. **B,** Similar spikelets after 15 days of growth. Bar = 10 cm.

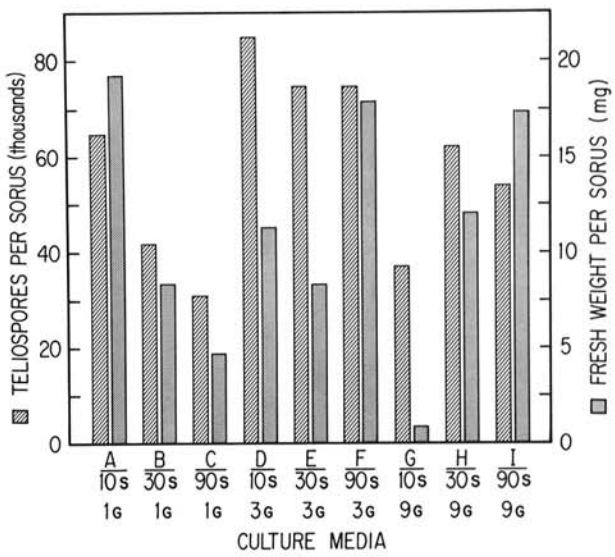


Fig. 7. Mean teliospores (*Tilletia controversa*) produced per sorus and mean fresh weight of each sorus in the primary basal floret in detached wheat spikelets, selection 77082, cultured for 16 days in various levels of sucrose and L-glutamine. Sucrose levels: 10 S = 10 g L^{-1}, 30 S = 30 g L^{-1}, 90 S = 90 g L^{-1}. Glutamine levels: 1 G = 1 g L^{-1}, 3 G = 3 g L^{-1}, 9 G = 9 g L^{-1}. $n = 4$, at $P = 0.05$; LSD = 23,500 (teliospores) and LSD = 5.5 mg (fresh weight).

per ovary in 16 days of incubation in the best in vitro medium. The exponential increase in the rate of spore production in the in vivo ovaries was not observed in the ovaries in the cultured spikelets, where the rate of spore increase was nearly linear during the incubation period.

DISCUSSION

General field observations have indicated that the changes associated with many smut diseases of cereals and grasses affect several important yield components (2,7). In common and dwarf bunt of wheat, these symptoms always appear together as a syndrome that is characterized by increased dwarfing, tillering, seed set, and greenness (7). The present quantitative study indicates that the dwarf bunt pathogen alters the development of infected florets, e.g., interferes with anther development, blocks pollen development and lodicule functioning but enhances chlorophyll synthesis, and enhances the development of floral primordia and unfertilized ovaries.

Alterations in the normal hormones, or levels of hormones, in wheat plants may be the basis for this syndrome. Some of these alterations suggest changes in endogenous cytokinin levels, and healthy wheat plants treated exogenously with zeatin riboside or isopentenyl adenosine developed dwarf bunt-like symptoms (9). Zeatin riboside, isopentenyl adenosine, and five other cytokininlike compounds were detected consistently in hyphae and culture fluids of *T. controversa* (9). One of these unknowns was also present at high levels in bunt-infected (*T. caries* and *T. controversa*) wheat kernels. Thus, some evidence suggests that cytokinins are involved in the bunt disease syndrome, but it is unlikely that the entire syndrome is due to changes in a single hormone. The quantitative data presented in this paper may encourage detailed studies of hormonal effects on a specific symptom, e.g., continued development of ovarian tissue into a seedlike organ without fertilization. Such studies may provide important clues about the physiological nature of seed development and apomixis.

Because cytokinins are not readily translocated (11), the levels of cytokinins produced by fungi in specific tissues may be related to the hyphal mass that develops in that tissue. Cytokinins enhance the transport of assimilates into the cytokinin-rich area (11), thus a major advantage of cytokinin production by a pathogen may be to ensure the continuous movement of nutrients to an area where the pathogen is developing. Although only a few plant pathogenic fungi have been reported to synthesize cytokinins, most are smut fungi (1,4,6,9). The production of cytokinins in the host may be characteristic of many fungal pathogens.

Excised wheat spikelets infected with *T. controversa* were successfully grown for 16 days in liquid culture media containing sucrose, glutamine, salts, and minor organic factors. Varying the composition of the medium affected the number of teliospores produced in the sori. The highest level of sporulation in the detached spikelets occurred in spikelet culture media containing glutamine at 3 g L^{-1}.

The spikelet culture technique affords control over the nutrient and hormone supply to the spikelets and may be used to study the effect of many variables on the development of the pathogen, the host, the wheat bunt diseases, and other cereal smut diseases. By eliminating the mediating influences of the other organs of an intact plant, these in vitro cultures of excised spikelets provide opportunities for more precise and difficult experimental studies in biochemistry, physiology, pathology, and genetics, e.g., the effects and mode of action of anti-fungal compounds or the mechanism by which temperature-sensitive genes control resistance to the bunt diseases. Compared with intact plants, these in vitro methods reduce variation and enhance replicated studies.

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