The Physiology of Germination of Tilletia Teliospores

Edward J. Trione

Agricultural Research Service, U.S. Department of Agriculture, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis 97331.

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ABSTRACT

The germination of teliospores of *Tilletia caries*, the common bunt fungus, was studied on 3% agar medium. The thick, tough, spore wall is impermeable to lipid solvents and takes up water very slowly. Factors that reduced the permeability barrier of the wall, such as hydrolytic enzymes or lipid solvents, stimulated the spores to germinate about 5 hr sooner than controls. The emulsifier Tween 20 and dimethylsulfoxide both enhance permeability but both had an adverse effect on germination. Many substances reported to activate the germination of dormant fungal or bacterial spores were generally ineffective in reducing the dormancy of teliospores. Hydrated teliospores incubated for 24 hr or longer at 2 C did not germinate as early as cells hydrated at 22 C, but they did germinate at a faster rate once germination began. Light did not appear to enhance germination of teliospores that required only 3 to 4 days to germinate.

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Additional key words: common bunt, dwarf bunt, temperature, light, self-inhibitors.

Teliospore germination, promycelial outgrowth, formation of primary sporidia, fusion of primary sporidia and germination of primary sporidia are possible focal points for the control of *Tilletia caries* (DC.) Tul., the cause of common bunt, and *T. controversa* Kühn, the cause of dwarf bunt. The germination and post-germination developmental stages may be the weakest link in the whole life cycle and thus, most susceptible to control measures.

Under optimum environmental conditions, the time required for germination of teliospores of common and dwarf bunt races varies from 3 days to 3 months, respectively. Although 3 days is a short germination time for bunt teliospores it is a long germination period compared to most fungus spores.

Germination of common bunt teliospores proceeds normally on water agar but is reduced on nutrient agars (6, 11). Water-soluble inhibitors of germination were detected in dwarf bunt teliospores and were thought to be the cause of the extreme dormancy (12). Trimethylamine was reported to be a germination inhibitor of common bunt teliospores (6). Blue light enhanced teliospore germination in common bunt races possessing dormant teliospores (6, 16). In contrast, Zscheile (22) demonstrated that light was not important for either germination or sporidial development in Race-1, *T. caries*, but was required for maximum germination of *T. controversa* teliospores. Attempts to enhance germination of dwarf bunt teliospores with acids, bases, plant growth regulators and heat shock treatments were unsuccessful (5). Although germination characteristics differ between common bunt races (11), knowledge gained from a detailed study of a single race should apply to other common bunt races as well. The experiments reported herein have attempted to increase the rate of teliospore germination by various chemical and physical methods and to study the nature of the dormancy of these spores.

MATERIALS AND METHODS.–Teliospores of the common bunt fungus, Race T-5, were obtained from field-grown wheat plants in the experimental plots of R. J. Metzger, Geneticist, USDA, Corvallis, Oregon. Spores were shaken from the broken sori, air-dried, and passed through a series of standard sieves to remove host plant residues. The spores were used within a year after their formation and their viability did not decrease during that time.

All media used in germination studies were adjusted to pH 6.5. Agar media contained 3 g agar, 5 mg penicillin G and 5 mg streptomycin sulfate per 100 ml. The antibiotics were included to reduce the bacterial contamination while permitting normal teliospore germination. Agar plates were seeded with teliospores by spraying an aqueous suspension of the spores to obtain a uniform spore density of 10 spores/mm^2. The thin film of moisture was quickly absorbed into the agar. Agar plates, seeded with teliospores, were incubated in the dark at 17 C ± 1 C unless described differently for individual experiments. Microscopic examination of germinating spores was made at 75X. Spores were considered germinated when the length of the promycelium equaled or exceeded the spore diameter. One hundred spores were counted in each of two areas on two plates, and the experiments were repeated at least twice.

RESULTS AND DISCUSSION.–*Factors related to the teliospore wall.*–A common explanation of dormancy is that a permeability barrier must be overcome before germination can proceed. The thick,

tough, three-layered cell wall (1) of the *Tilletia* teliospore is nearly impossible to break or crack by the usual laboratory instruments for homogenization and therefore may be an important factor in dormancy. We have determined that in laboratory dry storage common bunt teliospores contain only 2.5% moisture. The rate of water uptake by these spores is very slow when hydrated by stirring in water, reaching about 10% moisture after 24 hr and 30% moisture after 48 hr. Partial removal or hydrolysis of the spore wall may accelerate the rate of germination of these spores. Alternatively, substances known to act as carriers of solutes across cell walls and membranes may also facilitate germination.

Hydrolytic enzymes.—The outer wall of teliospores can be partly degraded gently with the aid of hydrolytic enzymes. Dry teliospores (50 mg) were placed into 10 ml of 0.01 M KH_2PO_4 buffer, pH 6. Enzymes (20 mg each of cellulase, hemicellulase, pectinase and lipase) were added and the mixture was stirred for 2 hr at 22 C. Proteolytic enzymes (20 mg each of papain and protease) were then added and the mixture was stirred for another 4 hr. Control spores were stirred in distilled water for this same period. The spores were filtered, washed thoroughly and sprayed onto water agar plates. After 72 hr of incubation, 73% of the enzyme-treated spores had germinated compared with 3% of the control spores. After 96 hr of incubation, 79% of the treated spores and 74% of the control spores had germinated. When tested individually, the enzymes had little or no effect in speeding up the rate of germination. Microscopic observations of the spores treated with the enzyme mixture indicated that the sheath and much of the outer reticulate wall had been removed, and the walls had the appearance of a dense sponge. In spite of this appearance, the enzymes apparently did not damage the plasma membrane of these spores for normal germination followed.

Organic solvents.—Teliospores contain lipoidal substances, including waxes, in the outer spore wall (10, 20). These lipoidal substances are probably involved in maintaining water balance in the spores but may also function as a barrier against the transport of polar substances into or out of the protoplasm of the teliospores. Removal of these substances should greatly increase the permeability of the spore wall.

Teliospores (100 mg, race T-5) were refluxed with 100 ml diethylether for 1 hr. The ether was filtered off and a small sample of spores was placed on water agar to test germination. The rest of the spores were placed in a fresh 100-ml of ether and refluxed again for 1 hr. This procedure was repeated until the spores had been extracted for five 1-hr periods. Maximum germination and primary sporidial formation on water agar were observed in all samples, including the spores that had been extracted for 5 hr. In subsequent experiments, teliospores were refluxed in ether for 24 hr with no loss in viability. The teliospores that were extracted with ether began to germinate 3 to 5 hr before the unextracted control spores. The final percentage germination of the ether-extracted and the control spores was the same. Teliospores were also found to germinate maximally after extraction with benzene: chloroform (3:1) at 50 C for 30 min followed by a second extraction with n-heptane at 50 C for 30 min. These experiments indicate that the teliospore wall is impermeable to these lipid solvents, for if the solvents had penetrated the wall, they would have destroyed the delicate endomembrane systems and killed the spores. The zone in the cell wall that blocks the solvents may contain a dense deposition of very polar compounds.

To provide some information about the polar permeability barrier, the effect of solvents of increasing polarity (indicated by the dielectric constant) on the viability of spores was studied. Teliospores (50 mg) were placed in 10 ml of each solvent, stirred vigorously to get a uniform suspension, and incubated for 1 hr at 40 C, 50 C, and 60 C. The spores were then filtered, thoroughly washed with water and sprayed onto a 3% water agar plate, and incubated at 18 C. The results, Table 1, indicate that the lower alcohols are quite toxic; whereas, the higher alcohols are not toxic even at 60 C for 1 hr. Except for glycerol there appears to be a good correlation between toxicity and dielectric constants > 25.

TABLE 1. The effects of organic solvents and temperature on teliospore germination percentage of *Tilletia caries*

Solvent	Boiling point (C)	Dielectric constant	Germination (%) temperature treatments		
			40 C	50 C	60 C
Water	100	78	34	4	0
Glycerol	290	42	54	58	67
Methanol	65	33	1		
Ethanol	78	24	28		
Acetone	56	21	32	54	
1-Propanol	98	20	43	37	47
2-Propanol	82	18	42	49	40
1-Butanol	117	18	36	50	42
Chloroform	61	5		50	

Emulsifying agents.—Many surface-active agents aid in the transport of solutes through cell walls and across membranes. If permeability were a major factor in the dormancy of these spores, perhaps such substances would enhance germination. The rate of germination of rust uredospores was increased when emulsifiers were placed in the bathing medium (2, 16).

Emulsifiers are commonly esters of fatty acids and range in chemical characteristics from the extreme nonpolar, lipophilic types to the extreme polar, hydrophilic types. By mixing several of these substances in different proportions, a series of intermediate solutions can be achieved which have different capacities for making stable emulsions of oils in water or vice versa. Preliminary experiments on the effect of surfactants on teliospore germination indicated that they not only effected germination but

TABLE 2. The effect of Tween 20 on teliospore germination percentage and promycelial length (*Tilletia caries*)

Tween 20, (μg/ml)	% Germination	Promycelium, μ
0	75	40
1	68	60
3	76	120
10	60	120
20	33	120
30	35	80
40	42	80
60	35	80
80	0	0
100	0	0

also profoundly effected the subsequent development of the promycelium.

Tween 20, a commonly available emulsifier, has been used in many rust uredospore germination studies (2, 16). Table 2 shows the results of an experiment in which filter-sterilized Tween 20 was added to the water agar. Concentrations of Tween 20 greater than 10 μg/ml had an adverse effect on percentage germination, but concentrations between 3 and 60 μg/ml had a stimulating effect on promycelial outgrowth. At Tween 20 concentrations greater than 1 μg/ml the promycelia did not produce primary sporidia but instead continued to grow and branch. Cytological examination of these promycelia treated with 1 and 10 μg/ml Tween 20 showed eight nuclei per promycelium indicating that Tween 20 did not block nuclear division. Primary sporidia were produced within 4 hr when germinating spores were removed from the agar containing the Tween 20, washed gently and plated on water agar without the emulsifier.

These observations may be of importance in understanding the physiology of binucleate vs. mononucleate hyphal growth. If the promycelium was prevented from reverting back to the mononucleate form (primary sporidia) by the concentration and type of surfactant, this phenomenon would be analogous to a susceptible-resistant type mechanism; for in a bunt-susceptible wheat plant only the binucleate form can develop, and the host is resistant to the mononucleate form. There are many naturally occurring surfactants that could function in this manner in wheat plants; e.g., water-soluble gums, phospholipids, lecithin-like compounds, colloids.

Additional experiments were performed with other surfactants and a range of solutions with intermediate hydrophilic-lipophilic values. Indications were that the concentration of surfactant was more important to this nuclear reversion phenomenon than the hydrophilic-lipophilic value. Some of these surfactants may have great value when combined with dimethylsulfoxide (DMSO) and seed treatment chemicals used in bunt control, for they would not only enhance spreading and penetration of the fungicides but would interfere with germination and some of the subsequent developmental stages of the bunt fungi.

Potential germination initiators.—Dormant fungus spores often fail to germinate when placed in conditions suitable for vegetative hyphal growth. Usually in these cases some type of physical or chemical activation is required. Chemical stimulants for germination appear to be either surface-active agents or substances that affect spore metabolism. Many have suggested that germination is an enzymatic process that is merely activated by germination agents such as alanine or adenosine. It has been shown, however, that germination of smut spores is not a simple process but, instead, consists of a complex series of metabolic processes (4).

Germination trials on *T. caries* teliospores were conducted, using substances that have been reported to activate or stimulate the germination of dormant fungal or bacterial spores. Octanol, octanal, nonanol, and nonanal are very effective, at micromolar levels, in stimulating the germination of wheat stem rust uredospores (2). They seem to function as anti-inhibitors, overcoming the natural endogenous inhibitors of the uredospore (2). Table 3 shows the effects of these substances on the percentage germination of common bunt teliospores, race T-5. These substances did not increase either the rate or the percentage germination. It is noteworthy that the two media which induce the most rapid germination of rust uredospores (2, 16) have no effect on these bunt teliospores. Thus, the physiology of germination of bunt teliospores appears to be regulated by different mechanisms than those occurring in rust uredospores.

L-alanine is a very specific triggering agent for the germination of many dormant bacterial spores (8, 9). L-proline stimulates the germination of some fungal spores (21). L-alanine and L-proline were incorporated separately into water agar at the following concentrations: 0, 1, 3, 10, 30, and 100 μM. They had no effect on the rate or the final percentage

TABLE 3. The effect of eight- and nine-carbon alcohols and aldehydes on teliospore germination percentage (*Tilletia caries*)

Concentration in water agar (μM)	Teliospore germination (%)			
	Octanol	Nonanol	Octanal	Nonanal
0	66	72	74	69
0.1	26	60	81	70
0.3	25	72	70	70
1	33	61	59	61
3	23	68	76	63
10	32	60	46	57
30	28	47	63	69
100	2	30	49	68
300	0	0	58	68
1,000	0	0	51	57

TABLE 4. The effect of calcium and dipicolinic acid on teliospore germination percentage (*Tilletia caries*)

Concentration in water agar (μM)	Teliospore germination (%)		
	$CaCl_2$	Dipicolinic acid pH 6.5	$CaCl_2$ plus dipicolinic acid
0	87	80	69
3	72	45	66
10	77	1	41
30	65	0	14
100	1	0	6

germination of T-5 teliospores. In addition, an amino acid mixture (Edamin, an enzymatically digested lactalbumin, Sheffield Chemical Co., Norwich, N.Y.), filter-sterilized into mineral agar (17), had little or no effect on germination or primary sporidial formation from 0.1 to 3.0 mg/ml. At 10 mg/ml, germination was only half that of the control, and primary sporidial formation one-quarter that of the control.

Many dormant bacterial spores can be triggered to germinate quickly if equimolar amounts of calcium and dipicolinic acid are placed in the medium (8, 9). Table 4 shows that the concentrations of calcium dipicolinate (10 to 30 μM), which are effective in stimulating germination of bacterial spores tend to inhibit the germination of common bunt teliospores.

Many fungal spores require an exogenous source of energy in the form of carbon compounds before germination will proceed. *Tilletia* teliospores do not require exogenous nutrients to germinate (6, 7, 11, 13). In germination trials with glucose added to water agar there was no effect on percentage germination, up to 0.5 M. However, at concentrations above 0.1 M glucose, the promycelia developed into long, thick structures and produced very few primary sporidia. At 0.5 M the promycelia developed into short, bulbous tubes that did not elongate and did not produce primary sporidia. The disaccharide, trehalose, a normal component of bunt teliospores, also had no effect on germination at concentrations up to 0.1 M.

DMSO increases the transport of solutes into tissues and also increases the leaching of low molecular weight compounds from tissues. DMSO has very low toxicity to many plants and fungi. DMSO was filter-sterilized and incorporated into water agar to give concentrations ranging from 0.1 to 20% DMSO. Teliospore germination was strongly inhibited at 1% DMSO and prevented at higher concentrations: 76% germination in the control; 68% at 0.1% DMSO, 45% at 0.3% DMSO, $<$ 1% germination at 1% DMSO. These results are in contrast to many fungi and plants that seem unaffected by 10% DMSO solutions.

Cyclic adenosine monophosphate (AMP) is an important activator of many key enzymes and has been found to function as a developmental hormone in some fungi (3). We hypothesized that cyclic AMP might aid in the activation of hydrolysis of endogenous storage materials in the teliospores and thus increase the rate of germination. Cyclic AMP, pH 6, was added via sterile filtration to warm water agar. The teliospores, Race T-5, were stirred for 2 hr in the same concentration of cyclic AMP as contained in the agar plate on which the teliospores were sprayed. The results demonstrated that cyclic AMP (from 1 μM to 10 mM) had little if any observable effect on teliospore germination.

Effects of temperature and light.—The majority of fungi that require cold treatment to activate their dormant spores belong to the Basidiomycetes. Little is known about the chemical transformations that take place in the spores during cold treatment. We do know, however, that in the cold, cellular membranes are in the expanded, "open" configuration, thus increasing permeability (14). Gases are more soluble in the cold, thus CO_2 and O_2 tensions increase. All these factors would tend to stimulate germination of *Tilletia* teliospores.

Tilletia teliospores germinate best at cool temperatures, but some species have an obligate requirement for cold treatments (5, 7, 13, 19, 22). Experiments were designed to determine if cold temperature enhanced germination of telisopores, Race T-5, which have an optimum of 18 C for germination (19). Teliospores were hydrated by stirring in water for 2 hr, filtered, washed thoroughly, and sprayed onto water agar. The plates were held at 2 C for various times and then transferred to an 18 C incubator. Other samples of teliospores were incubated for various times on a rotary shaker at 22 C in an aqueous solution of penicillin and streptomycin (each at 100 μg/ml). At the end of these treatments (up to 72 hr) on the shaker the spores showed no evidence of germination. These spores were filtered, washed thoroughly, sprayed on water agar, and incubated at 18 C.

The percentage germination of the teliospores was measured three times during incubation. Although the cold-treated cells did not germinate as early as the cells hydrated at 22 C, they did germinate at a faster rate once germination began (Fig. 1 and 2). Teliospores that remained at 2 C or 22 C showed less than 10% germination after 14 days. When compared with control spores incubated at 18 C, these data (Fig. 1 and 2) indicate that reactions favorable to germination proceed during the hydration and incubation at 2 C and 22 C. Figure 2 indicates that the germination process was closer to completion when the cells were incubated at 22 C compared to 2 C.

0% germination, it would be seen that the emergence of the germ tube from the teliospore began at about the same time, in an 18 C incubator, irrespective of the length of cold treatment. However, the rate of germination during the next 6 hr was directly correlated with cold treatment. Extrapolation of the solid lines in Fig. 2 to 0% germination indicate that the time of germ tube emergence was directly correlated with the time of hydration at 22 C.

The effects of temperature on the developmental

stages that quickly follow teliospore germination (promycelium outgrowth and formation of primary sporidia) were studied on a temperature gradient plate (19). Teliospores of *T. caries*, Race T-5, were suspended in distilled water, stirred for 1 hr, sprayed onto water agar plates and incubated at 18 C for 70 hr. The spores were then gently washed from the agar surface, filtered, washed and sprayed onto the water agar surface of the 20 X 20 cm plate which had a temperature gradient from 13 to 20 C. Strips of agar were removed periodically from the temperature plate and sprayed immediately with a cotton-blue-phenol solution to fix and preserve the germinating spores. These samples were examined microscopically and observations recorded on percentage germination, length of promycelium, percentage of promycelia that had formed primary sporidia, and the number of primary sporidia produced per promycelium. Maximum germination occurred at 16.5 C and temperatures between 17 - 20 C accelerated the rate of primary sporidial formation, with 50% of the germinated spores forming sporidia within 6 hr of plating. After 24 hr abundant primary sporidial formation was evident at all temperatures tested. Temperatures within this 13 to 20 C range had little effect on the length of the promycelium. Nutrients were not added to this medium so the germ tubes derived most of their energy from endogenous sources. All promycelia were between 20 and 25 μ long prior to primary sporidial formation. All observations indicated eight primary sporidia per whorl, irrespective of the agar temperature that existed during their formation.

Light has long been known to enhance the germination of very dormant *Tilletia* teliospores (13). Recent evidence (6, 15, 22) indicated that radiation in the blue and violet regions of the spectrum are most effective in stimulating germination of *Tilletia* teliospores. Irradiation from the near-ultraviolet (near-UV) fluorescent lamps (peak emission at 366 nm) has also been most effective in photoinduction of sporulation in many fungi (18). We hypothesized that the photoreceptors that function in sporulation may also be present in fungus spores and function in germination, hence such spores may respond to the same irradiation. Accordingly teliospores were hydrated and then irradiated with near-UV light for 1 to 8 hr beginning at 0, 24, or 48 hr during the germination process in an 18 C incubator. These light treatments had no effect on germination. The doses of irradiation used would have been photoinductive for sporulation of many species of fungi.

In another experiment teliospores, race T-5, were hydrated, kept in the dark until being placed in an 18 C incubator that had a diurnal 12-hr dark, 12-hr near-ultraviolet light regime, and remained there for the duration of the experiment. The germination of these spores was compared with that of spores incubated in continuous dark or continuous near-ultraviolet light. The results indicated that the various light treatments had little or no effect on the germination. As the lamps used in this study emit radiation up to about 440 nm (blue region), the

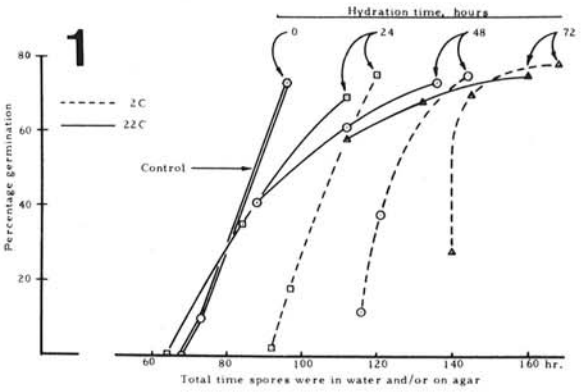

Fig. 1. The effect of hydration time at 2 C and 22 C on the percentage germination of common bunt teliospores.

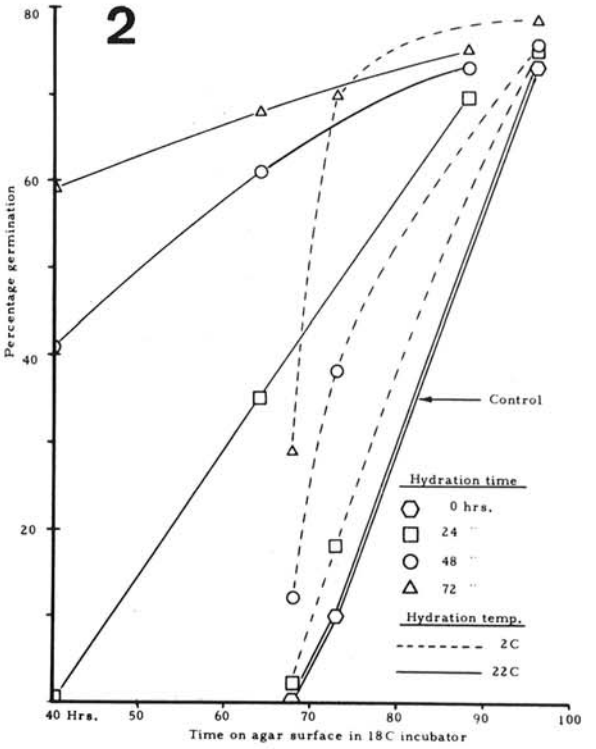

Fig. 2. The effect of hydration time at 2 C and 22 C on the percentage germination of common bunt teliospores on an agar surface at 18 C.

results suggest that irradiation from about 300 to 440 nm is ineffective in stimulating germination of race T-5 teliospores. These results agree with the results of Zscheile (22) but contrast to the results of Schauz (15). Schauz, however, used a race of *T. caries* whose teliospores took 5 to 6 days to germinate, compared to 3 days for teliospores of race T-5 used in the present study. The reported enhancement of germination by low intensity light is surprising in consideration of the thick, dark wall of *Tilletia*

teliospores. Most studies did not employ precise temperature control, and possibly the increase in temperature of the dark spores under illumination may have influenced the results in an unaccounted manner (22).

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