The Controlled Experiment in the Scientific Method with Special Emphasis on Biological Control

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The experimenter questions, manipulates, alters, controls, and forces nature to reveal herself. An experiment is a game in which phenomena are carefully watched, at a particular time and place, in relation to an intentional disturbance occurring in them and around them. According to Egler (6), "a controlled experiment is one where two or more situations are watched, where one is observed without interference, and where the others are manipulated in known ways. The changes are then related to this known manipulation."

The control is such an integral factor in the scientific method that it is taken for granted and treated only briefly, if at all, in the philosphical literature and is only rarely defined. Indeed, in most disciplines, a control is readily identifiable; for plant pathologists, however, establishing controls is complicated by the multitude of interacting factors in the classic disease triangle. The experiments involve pathogens; therefore, there can be inoculated and uninoculated controls. When plant pathologists study biological control, they add a new set of parameters (3) involving the introduction of one or more living entities. Below ground level, these living entities may be in various ecological habitats such as the raw soil, the rhizosphere, or in association with organic matter. To attempt to contrive controls for every possible interaction can disintegrate the science into folly.

A recurring theme in studies of biological control provides an illustration of the complexity of designing appropriate controls for experiments. Antagonists are often introduced into a plant disease system, for example, to induce suppressiveness in soil (4). Such microorganisms, however, are grown in pure culture and manipulations of supporting medium and/or substrate become important factors in the growth and reproduction of thalli (13) especially in commercial production (11). Thus, a solid substrate, like a peat-bran mixture (8,19), that has supported the growth of the agent, may be inextirpably associated with the microorganisms. After growth on this medium, the culture and substrate are dried, ground to a powder and then added to soil. After suitable incubation, plant and/or pathogen responses, if any, may be observed. There is considerable confusion among some researchers as to what constitutes an appropriate control or controls in this case. Some reviewers and editors of scientific journals have criticized any experiments described in manuscripts that do not have a "peat-bran control." We disagree with this reasoning and analyze, below, the various manipulations that have been suggested as appropriate "controls."

CANDIDATE CONTROLS

Use of sterile peat bran. By reference to the definition of a control, cited at the beginning of this article (6), it is apparent that the raw untreated soil is an appropriate control—this is the element "observed without interference"; but what is the nature of the "peat-bran control" in this case? The sterile peat-bran medium not infested with the biological control agent is a candidate. Yet, it is not the same substrate as that acted upon by the agent during incubation. Further, the addition of previously undigested organic matter to soil can induce increased disease severity and/or toxin production resulting from the activity of soil microflora leading to plant damage (15) or, alternatively, can lead to biological control (2,3). Sterile peat-bran medium has its own "interference" and is not an appropriate control.

Elimination of the biocontrol agent after growth. To allay these difficulties, perhaps the agent could be grown in the sterilized peat-bran medium and then autoclaved or fumigated to remove the living portion of the substrate. Much of the nutrient in the peat-bran would be utilized by the microorganism prior to the second sterilization; however, the dead cells, stable products extracted by the fungus during growth, and any residual unutilized nutrients would be present. Further, Bruehl (5) assured us that possession of a substrate by a soil microorganism is "nine points of the law" in the furiously antagonistic milieu that is soil. Obviously, even though the agent has acted on the substrate before sterilization, it is not identical in physical, chemical, or ecological properties to the substrate occupied by the living microorganism—it is not an appropriate control.

Infestation of the substrate. If possession of the substrate by a living entity is essential for a "peat-bran control," it might be possible to culture a microorganism that has all the attributes of the agent except the factor that is responsible for the response. This strategy was suggested as being most appropriate some time ago (1). Unfortunately, mechanisms are poorly understood and identification of that factor upon the initiation of an experiment is usually impossible. Perhaps the only available article that adheres to such a dictum is that by Kloepper and Schroth (12) in which mechanisms of iron competition were identified by comparing the activity of siderophore-producing pseudomonads (the treatment) with a mutant producing no siderophores (the control). If the mechanism is not known, however, perhaps the substrate could be occupied by the general soil microflora found in conducive soil. The peat-bran mix, then, is "seeded" with a small amount of raw soil and incubated just as in the treatment—an easy method but not excellent. Again, the specter of phytotoxins (15) produced by such a rampant microcommunity and transferred to the soil distorts the information demanded of a control. Alternatively, a reduction in disease is possible from such a manipulation. Rao and Rao (16) added inoculum growing on substrate to soil at various densities. At relatively low densities a typical inoculum density-disease curve was generated but at higher concentrations, the more substrate-inoculum added the lower the disease incidence. It was concluded that the substrate added with the inoculum actually induced biological control at the higher application rates. Clearly, introducing microorganisms indiscriminately for the purpose of colonizing a substrate is not a control.

The biocontrol agent without substrate. Is it possible to add the biological control agent itself without the medium to isolate the

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effect of the substrate and, thus, generate a control? Prima facie this could be an appropriate control in a positive sense; however, quantitative considerations make the approach untenable. Conventionally, population densities are measured in colony-forming units (cfu) on selective media. Many propagules embedded in a single unit of powdered peat-bran mixture would yield only one cfu; single propagules would also yield one cfu. When propagules are added to raw soil, a substantial decrease in propagule density usually occurs (2). In contrast, even if a proportion of the propagules embedded in a substrate are rendered nonviable, the unit would still yield a cfu. Again, propagules embedded in the particle of peat-bran would have increased inoculum potential (sensu Garrett [9]) over the single propagule without substrate. Clearly, manipulations to isolate the activity of the agent without substrate on an equal quantitative basis are impossible without extensive experimentation to determine inoculum potential relationships, ie, such a strategy is not a control.

EXPERIMENTAL ANALYSIS

In the literature of plant pathology, there is extensive experimentation reported pertaining to chemical control. Few fungicides are applied in a pure state but are formulated as dusts, water-dispersible powders, emulsions, or emulsifiable concentrates. Diluents or carriers are seldom applied in the controls and the reasons for this may be related to the principles treated above. Nevertheless, the various recommended "peat-bran controls" (elaborated above) were incorporated into an experiment to test the theoretical basis upon which the principles were constructed.

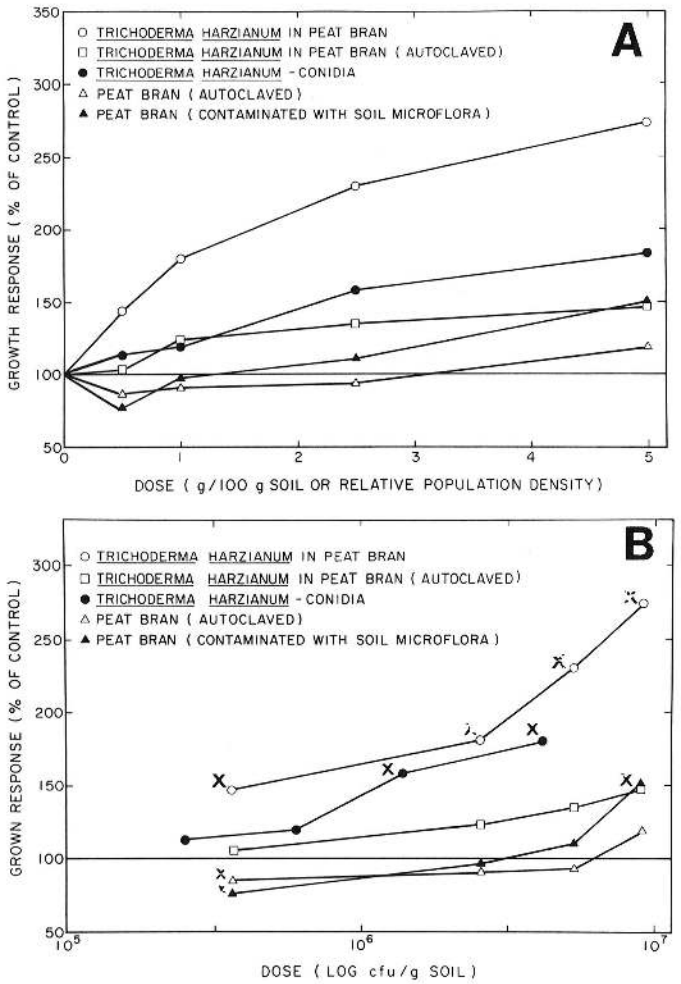

Fig. 1. The effects of *Trichoderma harzianum* previously grown in peat-bran medium applied at various dosages and various manipulations of this treatment on the oven dry weight of leaves of radish after 6 wk of incubation. Each treatment at each dosage contained five replications and there were 10 plants per pot. **A,** Dosages were plotted according to weight of peat-bran or as relative amounts of conidia added on the absissa as appropriate. **B,** The same data plotted by matching colony-forming unit dosages on the absissa. By regression analysis, there were no significant differences ($P = 0.5$) in slope values among the various manipulations. When means of each manipulation were compared, only *T. harzianum* grown in peat bran was significantly different. A × identifies points that are significantly different ($P = 0.05$, FLSD) from the control (the horizontal line at 100%) at each level of application.

We used *Trichoderma harzianum* Rifai to increase the growth of many plant species. Such responses by use of antagonists is thought to be mediated by processes associated with biological control (18). We exposed radishes (*Raphanus sativus* L. 'Early Scarlet Globe') to the various manipulations listed in Fig. 1 and the elements added to raw soil (characteristics given in reference 17) were incorporated at various concentrations. A balanced fertilizer solution was applied at each irrigation to ensure that nutrients were not limiting to plant growth. After 7 wk, various measurements of the growth of the plants were made; although Fig. 1 presents only the average dry weight of leaves produced, this reflected the trends of other characteristics used in measurements.

The first problem in plotting the data was that of quantifying the various manipulations. Fig. 1A was plotted according to relative amounts of substrate in the various materials added to the soil. Conidia of *T. harzianum* were added at multiples of 10^6 conidia per gram of soil. Obviously, this does not match the population density of the various manipulations of the added (viable) thallus units of *T. harzianum*. Therefore, Fig. 1B plots and matches data according to the cfu (7) observed 1.5 wk after the initiation of the experiment in the *T. harzianum*-peat bran or *T. harzianum*-conidia manipulations. Manipulations not involving *T. harzianum* are plotted according to equivalent weight of substrate added initially. The following inferences, therefore, are based largely on Fig. 1B. Statistics to determine differences among the manipulations were applied according to regression analysis (10). To determine whether statistical differences ($P = 0.05$) existed at each application rate, Fisher's least significant difference analysis (14) was applied.

There was a significant increase in dry weight of leaves of radishes grown in the presence of *T. harzianum* cultured in peat-bran medium, as compared with those grown in raw soil, at all levels of application from 3.8×10^5 to 9.1×10^6 cfu/g soil. As the concentration increased, plant growth increased to 274% of the control at 9.1×10^6 cfu/g soil. When the peat-bran culture was autoclaved, there was a small, but significant, increase in dry weight compared with the raw-soil control at higher application rates. This suggests that such a "peat-bran control" could yield differing results depending on the quantity and/or quality added to soil. Indeed, in previous work at Colorado State University, we found no significant increase in radish growth in soil amended with substrate as compared with growth in raw, unamended soil; whereas, in experiments at the Hebrew University, small but significant increases were observed.

When peat bran was added either infested or noninfested with the soil microflora, growth was less than in raw soil at low levels; at the highest concentration, however, growth was greater than in the raw soil.

Regression analysis did not reveal any significant effect on growth of radish by the addition of conidia without substrate to soil. However, comparison of values at each population density show that this manipulation induced growth at a significantly higher level than the control at the higher densities. The necessity for transformation of data values, which may or may not be valid from an objective and/or a statistical standpoint, illustrates the difficulties inherent in using this type of "control."

CONCLUSIONS

The results are partially explained by the principles involved in the ecology of the soil microbiology briefly elaborated above. Responses were variable, and their magnitude depended upon dosage levels. It is apparent that *T. harzianum* applied without substrate was capable of initiating an increased growth response; however, other manipulations not involving the agent also induced

the response. Thus, there were multiple causal factors operating depending on treatment.

Therein lies the crux of the concept of what constitutes an appropriate control in such an experiment. All the manipulations are treatments, not controls; in every case except the raw soil control, there was interference. A "peat-bran control" is impossible. Therefore, the critical decision of what constitutes the control is the question asked of the experiment. Did treatment with the *T. harzianum*-substrate induce increased growth over an untreated control? The answer is yes. Does treatment with conidia of *T. harzianum* induce a similar response? Yes, but of lower magnitude (at equivalent levels of cfu). Since the living agent (without substrate) induced the response, this suggests that it was mediated by interactions in the biology of the system. Do other treatments influence growth? In some cases, yes, but responses are variable according to dose. If used as controls, these treatments tell us little about causal factors; the appropriate control in all cases is the manipulation without interference—the raw soil control.

This does not belittle the value of experiments including such treatments. Certainly, useful information can be obtained with these manipulations.

Proper identification of such manipulations as treatments rather than controls goes beyond simple semantics. If the so called "peat-bran control" is not a control but a treatment, then the data accumulated by use of this strategy should yield definitive results. When adequate parameters have been established by acceptable scientific procedures, the principles developed can be used as a basis for other experiments. If such manipulations are misidentified as controls, however, they must be used in every experiment in that category adinfinitum even though they are not appropriate.

Blind application of the elements of the scientific method is not a substitute for imaginative thought. "Concepts are games we play without heads; methods are games we play with our hands" (6). In identifying controls, concepts are appropriate before the hands are employed.

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