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An integrated approach to simultaneously control insect pests, powdery mildew and seed borne fungal diseases in barley by bacterial seed treatment

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Abstract: In connection with extensive testing of a large number of bacteria for biological control of fungal seed borne pathogens, we have noted on several occasions that application of these bacteria to seeds also protects against insect pests and diseases attacking acrial plant parts. Thus a single treatment can offer the plants protection against a large variety of agents. The method of applying bacteria to seeds of cereals has been developed for large scale commercial use by BioAgri AB (Uppsala, Sweden), for example in the product Cedomon™. Twenty eight diverse bacterial strains representing diverse taxa isolated from different Swedish and Moroccan soils were tested for induction of resistance to powdery mildew in barley (*Blumeria graminis* f. sp. *hordei*). Seeds coated with bacterial culture broth resulted in plants that were either more susceptible or more resistant to powdery mildew. The four strains that induced the highest level of resistance to powdery mildew were tested more stringently in repeated tests. They were also tested for protecting barley plants from visitation of leafhoppers (*Psammotettix alienus*). A pot containing plants that had emerged from untreated seeds was placed in the same insect cage as one that contained plants that had emerged from treated seeds. The pots had no physical contact, even through irrigation water. Several leafhoppers were placed in each cage when the plants were 10 days old. The feeding preference of the leafhoppers toward plants emerging from treated vs. untreated seeds was recorded over a 14 day period. The results from repeated greenhouse trials indicate that certain strains of bacteria have a great potential not only to control seed borne diseases of cereals, but also to induce resistance to leafhoppers and powdery mildew, whereas others only induce resistance to leafhoppers. The diversity in the response of plants to bacterial seed treatment is intriguing. Ongoing field trials are assessing the persistence of this resistance response under field conditions. The most effective strains will be candidates for commercial development of multipurpose biocontrol agents.

Key words: induced resistance, biological control, *Pseudomonas*

Introduction

There is a large number of microorganisms in the rhizosphere and phyllosphere of plants whose importance to the ecology, growth and condition of plants is not very well known (Gerhardson & Wright, 2002). Some of them can induce enhanced plant growth and resistance to diseases caused by pathogenic bacteria or fungi (Kloepper et al., 1993; Van Loon et al., 1998), whilst others induce resistance to insect pests (Zehnder et al., 1999; Ramomoorthy et al., 2001). Many of the growth enhancing bacteria are referred to as PGPR (plant growth promoting rhizobacteria) (Buchenauer, 1998).

Resistance can be mediated through distinct signaling cascades in the plant – despite the biological end result being the same – depending on the type of signal or stimulus that is produced by the inducing bacteria. "Systemic acquired resistance", abbreviated SAR (Kuc, 1983, 1987), is induced in response to agents such as plant pathogenic microorganisms, where salicylic acid is a central intermediate (Kessmann et al., 1994). Induced systemic resistance (ISR) is a term that has been employed to describe a pathway to resistance that is mediated through nonpathogenic microorganisms, and for which jasmonic acid is an intermediate (Van Loon et al., 1998). SAR is known to mediate resistance to insect pests as well as various plant pathogens (Inbar et al., 1998; Hammerschmidt & Kuc, 1995). Zehnder and coworkers (1997a & 1997b) treated seeds of cucumber and soil for sowing with PGPR and noted a significantly lower level of attack of leaf beetles (*Acalymma vittatum* and *Diabrotica undecimpunctata*) in the plants whose seeds had been treated with bacteria. The first trials were conducted in greenhouses for short periods of time. However, in field grown cucumber whose seeds had been treated, the effect was observable close to two months after field planting of seedlings. The resistance observed in these trials was tentatively designated ISR (Zehnder et al., 1999). In our collection of microorganisms we have nonpathogenic, environmental isolates of bacteria that have been screened and selected for their biocontrol properties to Fusarium diseases of wheat (Johansson et al., 2003) and barley leaf spot (Hökeberg et al., 1997). We describe below how some of these isolates also have the ability to induce resistance in barley to feeding of leafhoppers (*Psammotettix alienus*) (Dahlbom) and to incidence of leaf rust (*Puccinia hordei*) and powdery mildew (*Blumeria graminis* f. sp. *hordei*). Powdery mildew is considered to be one of the most important fungal diseases of barley (Wiik et al., 1995).

Materials and methods

Bacterial strains, barley cultivars, fungal isolates, insects, growth conditions and media

The following bacterial isolates were used in this study: *Pseudomonas chlororaphis* strain MA342 (Hökeberg et al. 1997); several pink pigmented facultative methylotrophic bacterial isolates from Morocco: MA2, MA3, MA4, MA5, MA6, MA7, MA8, MA9, MA10, MA11, MA14, MA15, MA16, MA18, MA19, MA22 and MA23 (Saad Omer, 2004); and isolates MF30, MF174, MF181, MF200, MF231, MF299, MF304, MF400, MF417, MF434 and MF588 (Johansson & Wright 2003) isolated in Sweden and Switzerland. The bacteria were grown routinely on Tryptic Soy Agar (TSA) (Difco, Becton Dickinson, Le Pont de Claix, France) at 22°C. For seed application, the MF isolates and MA 342 were grown in Tryptic Soy broth (Difco) for 48 hours at 19 to 22°C. Prior to seed application, the Moroccan (MA) isolates were cultivated on TSA plates at 27°C for five days. The bacterial growth on each plate was resuspended in 10 ml tap water. For powdery mildew trials, the two near isogenic lines of the cultivar 'Pallas' P01 and P02 (Kølster et al., 1986) were used. They have race specific resistance to the powdery mildew races A6 and C15, respectively. These races were used in the experiments. The barley cultivar 'Ingrid' is susceptible to leaf rust and was used to test induction of resistance to leaf rust. The rust isolate used was ND97-21. It originates from Brian Steffenson, Univ. of Minnesota, St. Paul and was kindly provided by Dr. Anders Falk, Dept. of Plant Biology and Forest Genetics, SLU, Uppsala, Sweden. Leafhoppers of the species *P. alienus* were collected in the field (Azrang, 1976). They were maintained at 25°C in a ventilated rearing cage in the greenhouse. For leafhopper experiments, three to four leafhoppers were removed from the rearing cage with a suction apparatus and placed in an experimental cage consisting of a stocking that had been placed over bent wires over a pot. Leafhopper experiments were carried out at 25°C. The leafhoppers were placed on plants of 1 decimeter length (2nd leaf stage) and their feeding preference was recorded four times a day for a period of 10 to 14 days.

Bacterial application

A suspension containing approximately 10^8-10^{10} cfu/ml, were applied to the seeds, in accordance with the established procedure (Hökeberg et al., 1997). Treated and untreated seeds were sown in separate pots. The pots were placed in the greenhouse at 23 to 25°C.

Powdery mildew and leaf rust trials

Forty eight hour broth cultures of the 28 isolates listed above (MA and MF series) were applied to seeds (150 ml culture broth per 150 seeds). These isolates were screened for the ability to induce resistance to powdery mildew in P01 and P02. When the plants had reached a height of 1 decimeter, they were challenged with powdery mildew. Conidia were shaken over the first leaf of emerging barley plants. The number of spores per leaf area was counted according to the method described by Thordal Christensen & Smedegaard Petersen (1988). Methods for inoculation of powdery mildew and scoring of symptoms are described in Cho & Smedegaard Petersen (1986) and in Haugaard et al. (2001). The inoculated plants were placed at 19°C in the greenhouse. The number of powdery mildew colonies per 4 centimeters of leaf length was counted for six pots and five leaves per pot for every treatment. For leaf rust experiments, the leaves were spray inoculated with a method that we developed (data not shown) when the leaves had reached a height of one decimeter. The plants were covered with plastic overnight. When initial rust pustules appeared, leaves were removed and weighed, and the number of spores per mg leaf was estimated. Spores were washed from the leaves and suspended in tap water containing a droplet of soap and counted in the microscope.

Statistical analysis

The data were analyzed with Anova and differences between means of the treatments were tested with Duncan's multiple range test at $P \leq 0.05$.

Presence of bacteria in aerial plant parts

The presence of bacteria in aerial plant parts was checked. Seeds treated with isolates MF231, MF299, MF304 and MF417 were sown in separate pots, five pots per treatment. Untreated seeds were sown as a control. The plants were grown at 20°C. When the plants had reached a height of 1 decimeter, a leaf disk from one leaf per pot was taken at a random location, surface sterilized in 70% ethanol and crushed with mortar and pestle in 1 ml $MgCl_2$. The suspension was serially diluted and plated on TSA with or without supplement of cycloheximide.

Results and discussion

Plants originating from seeds treated with some of the isolates were less attractive as a source of food for *P. alienus*. The mean visitation frequency was significantly greater on untreated plants than on plants whose seeds were treated with the following isolates: MA 342, Ki95, MV94 and MF29. These isolates were known from previous work to provide protection against fungal seed borne diseases of cereals (Johansson et al., 2003; Borowicz, 1998). The Moroccan isolates (MA isolates) and MF30, MF174, MF181, MF200, MF231, MF299, MF304, MF400, MF417, MF434 and MF588 were not tested for induction of insect resistance.

Among the MA isolates, MA14 consistently provided protection from powdery mildew *(in two tests)*. Seeds treated with MA14 produced plants that were significantly less attacked by the fungus than those of the other treatments and that of the untreated control. There was less incidence of powdery mildew on plants that had originated from seeds treated with MF299, MF231, MF304 and MF417. The reduction of powdery mildew incidence ranged from 29 to 44% for MF231, from 21 to 63% for MF304 and from 31 to 38% for MF417. Similar results were obtained on several occasions, and these four isolates were selected for

subsequent field trials. Isolate MF304 was the most effective of the MF isolates in providing protection against leaf rust.

We attempted to isolate bacteria from aerial plant parts to ascertain that the effect we saw was not due to a direct interaction of the isolates with the powdery mildew fungus, the rust fungus or the leafhoppers. Suspensions containing surface sterilized and crushed leaf disks did not contain any traces of the bacterial isolates that had been applied to the seeds. In another experiment, seeds were coated with a version of strain MA 342 that had been tagged with a double copy of the gene for green flourscent protein (Tombolini et al., 1999). Nonsterile, crushed barley leaves were plated on kanamycin containing medium, and no bacteria were recovered then either (data not shown). These results indicate that the bacterial isolates do not travel through the plant nor in any other way come in contact with the pathogens or insect pests used as challenge treatments. It is therefore likely that the bacterial isolates of this study are inducing a resistance response in the plants.

Several other factors, such as plant growth regulating substances, herbicides, mechanical damage, BTH (a synthetic form of salicylic acid), jasmonic acid and some insect pests (Kogan & Paxton, 1983; Fischer et al., 1990; Green & Ryan, 1972; Inbar et al.,1998; Thaler, 1999; Stout et al.,1998) have also been reported to induce resistance. Induction of resistance to powdery mildew has been documented by several research groups, for instance by Smedegaard Petersen's group in the mid 1980's (Cho & Smedegaard Petersen, 1986; Thordal Christensen & Smedegaard Petersen, 1988). These researchers managed to induce a high level of resistance by first treating the barley leaves with (incompatible) virulent and avirulent forms of *B. graminis* f. sp. *hordei*. This pretreatment protected the leaves from the subsequent challenge with a virulent (compatible) race of the fungus. Their results were impressive. However, it is difficult to imagine how this method could be developed for use in practical agriculture. In the case of the microorganisms described in this work, it is conceivable that they could be developed for use in commercial agriculture, since seed application methods for large scale production have been developed. In the future, when field efficacy of isolates has been established, we foresee the development of this work for use in practical agriculture.

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