# Annual Report
# to
# The United States Golf Association

from


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## I. Executive Summary of the Proposal

The major problem associated with creeping bentgrass are pathogenic diseases. Most pathogens contain chitin in their cell walls, and therefore may be susceptible to chitinases [1]. The objective of this project was to develop fungal disease resistant creeping bentgrass/Penncross (*Agrostis palustris* Huds.) plants, initially by introducing a chitinase gene in this plant. However, after being funded we realized that one single gene may not necessarily work, and pathogens may develop resistance against a single gene product within a short period of time. Therefore, we supplemented four fold research to our proposed project. These research lines include (1) introducing a chitinase gene as proposed, (2) introducing a protease inhibitor gene in plants (because proteases which are essential for the survival of the pathogenic fungi ) , (3) introducing a drought resistance gene in plants (because less need for irrigation would prevent growth and spread of diseases), and (4) introducing a bialaphos resistance gene to creeping bentgrass (because should plants become resistant to bialaphos-herbicide, bialaphos would simultaneously control weeds as well as certain pathogenic diseases).

During last year, we followed up on introducing a chitinase gene, a protease inhibitor gene, the bar (bialaphos resistance) gene, and a drought resistance (mannitol dehydrogenase) gene into creeping bentgrass.

As we reported earlier, we previously developed transgenic creeping bentgrass which express a potato proteinase gene and the bialaphos-herbicide resistance genes. At present, these transgenic plants are in the field. The herbicide resistance of these transgenic plants were confirmed a long time ago. Within last year, we examined these transgenic plants after they were sprayed with biolophos for their resistance to dollar spot, brown patch and *Pythium*. The results confirmed that after we spray transgenic creeping bentgrass with biolophos (Liberty herbicide), we simultaneously control weeds, dollar spot and brown patch diseases at the greenhouse level. This experiment will be repeated at the field level by Dr. Vargas next summer. We have also introduced the chitinase gene and a drought resistance gene to creeping bentgrass. The selectable marker for this experiment has also been the bialaphos resistance gene. All plants regenerated are bialaphos resistance. Since in our construct, the herbicide resistance gene was linked to the drought resistance gene, we are sure that these transgenic plants also contain the drought resistance gene. Hundreds of transgenic plants have been produced from the chitinase/drought resistance experiment. Work is in progress to confirm the stable integration and expression of the chitinase gene and the drought resistance gene in these plants.

Eventually, all these transgenic plants must be cross bred and tested for their resistance to the major pathogenic diseases.

# ANNUAL REPORT

## II. Introduction

Dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*) and Pythium blight (*Pythium aphanidermatum)* are major pathogenic diseases of turfgrass.. Also, all of these pathogens contain chitin. The Sticklen laboratory team has cloned and characterized a full length chitinase gene which contains the necessary chitin-binding domain ([2; 3; 4] Gene Bank Number L22032). This laboratory also has constructed several plasmids containing a potato proteinase inhibitor II controlled by different (wound-inducible and constitutive) promoters. She has also obtained genes for a drought resistance and the bialaphos-herbicide resistance from other laboratories. During the last six months, an enormous amount of research was performed as follows.

## III. Genetic Engineering of Creeping Bentgrass with Chitinase and a bialaphos-herbicide Resistance Genes

A. Callus initiation and co-transformation
The protocol developed in Sticklen's laboratory for regeneration of creeping bentgrass [5] was used to produce embryogenic callus from caryopses of creeping bentgrass. Furthermore, the protocol which also was developed in her laboratory for genetic engineering of creeping bentgrass [6] was used to introduce the pHS2 chitinase gene and the bialaphose-herbicide resistance gene to this plant. For genetic engineering of creeping bentgrass, we used a plasmid containing our plant chitinase gene (pHS2 [7]) and a plasmid (pJS101 ) containing the herbicide resistance and a drought resistance gene (mannitol 1-phosphate dehydrogenase). Co-transformation was carried out by bombarding embryogenic callus with microprojectiles coated with a mixture of the two plasmids. Since there has been no report of using co-transformation method in creeping bentgrass, this project will also provide some important data in terms of the efficacy of co-transformation in creeping bentgrass. Within last year, a total number of 300 petridishes (over 1000 gun shots) have been bombarded with these two gene constructs.

B. Selection for bialaphos resistant calli
All of the above callus lines were selected for their resistance to bialaphos. Herbicide resistant calli have been selected at 5 mg/l and 10 mg/l of bialaphos. Selection has been carried out by subculturing the fast-growing, light -colored calli on the selective medium with 5 mg/l bialaphos at the interval of 14 days and then on the medium with 10mg/l bialaphos (for 2 times of subculture). A cycle of 3 months was needed to complete the selection process for the bombarded callus culture. Until now, three lines of bialaphos resistant callus have been selected, while more newly bombarded material has been put under selection.

C. Plant Regeneration
Regeneration of the putatively transformed plants has been undertaken by placing the bialaphos resistant calli on the MS medium with 10 mg/l of bialaphos under light. Green leaf primodia were observed within two weeks of culture. Approximately 40

days of culture in the petridishes were needed before the plantlets contained fully developed roots and were transferred into greenhouse pots. One of the three lines of bialaphos resistant callus, named 9601, has already been regenerated and transferred into greenhouse. The regeneration of the other two lines is under the way.

D. Confirmation of gene integration-----Southern blot
The genomic DNAs of the several lines as well as the untranformed Penncross have been isolated from the leaves of putatively transgenic plants. Southern blotting is in progress to confirm that the chitinase gene has been incorporated in creeping bentgrass.

**IV. Simultaneous control of weeds, dollar spot and brown patch diseases in herbicide resistant transgenic creeping bentgrass**

As reported last Fall, our transgenic creeping bentgrass expressing both the bialaphos herbicide resistance gene and the potato proteinase inhibitor II gene are in the field. We examined the simultaneous resistance of these plants to bialaphos-herbicide and to three pathogenic diseases of this plant. The details of our work are explained below.

A. In vitro test of fungal pathogens using bialaphos and PPT
Fungal pathogens *Rhizoctonia solani*, *Sclerotinia homoeocarpa*, and *Pythium aphanidermatum*, the etiologic agents of brown patch, dollar spot, and *Pythium* blight diseases, respectively, of creeping bentgrasses were inoculated and cultured on PDA medium supplemented with various concentrations of bialaphos or PPT to assess their responses to these two inhibitors of glutamine synthetase.

*Rhizoctonia solani* was very sensitive to the addition of bialaphos into the PDA medium. Even at the lowest concentration of one mg/l, the mycelial growth of *Rhizoctonia solani* was significantly suppressed as compared to that on PDA medium with no bialaphos supplement. Only about five mg/l (ED50 = 5.54 mg/l) of bialaphos amendment was needed to reduce the fungal growth by 50 %. There was almost no fungal growth observed four days after the inoculation when the bialaphos concentration of PDA medium was 60 mg/l. There was still no significant growth of *Rhizoctonia solani* even two weeks after the initial inoculation when the concentration of bialaphos was 60 mg/l or higher (data not shown).

PPT (the precursor of bialaphos) was also very effective in the suppression of the mycelial growth of *Rhizoctonia solani*. However, the presence of PPT was not as effective as that of bialaphos in suppressing the growth of *Rhizoctonia solani* on PDA medium . bialaphos inhibited mycelial growth of *R. solani* more than PPT did, as reflected by their values of ED50. The growth of mycelium was significantly reduced at the concentration of 25 mg/l PPT. More PPT amendment (292.18 mg/l or 1475.66 17.10 μM), as compared to the amount of bialaphos supplement (5.54 mg/l or 17.10 μM), was necessary to reduced the growth of *Rhizoctonia solani* by 50 %. There was still some mycelial growth of *R. solani* observed even when 600 mg/l of PPT was amended into the PDA medium. The same trend was also evident for *Sclerotinia homoeocarpa* and *Pythium aphanidermatum*, where the ED50 values for *S. homoeocarpa* and *Pythium aphanidermatum* were higher for PPT than for bialaphos.

The mycelial growth of *S. homoeocarpa* was also sensitive to the presence of bialaphos and PPT, though its responses were apparently different from those of *R. solani*). Higher concentrations of bialaphos and PPT were necessary to significantly reduce the mycelial growth of *S. homoeocarpa* than that of *R. solani*. The ED50 value of *S. homoeocarpa* for bialaphos was higher than that of *R. solani* (33.04 and 5.54 mg/l, respectively). More than 150 mg/l of bialaphos amendment was necessary to completely suppressed the mycelial growth of *S. homoeocarpa* on PDA medium. However, *S. homoeocarpa* responded a little more sensitively to higher concentrations of PPT (between 400 and 600 mg/l) than *R. solani* . The ED50 value for PPT of *S. homoeocarpa* was lower than that of *R. solani* (270.06 and 292.18 mg/l, respectively). In general, the effect of bialaphos or PPT amendment into PDA medium on the inhibition of mycelial growth of *R. solani* and *S. homoeocarpa* was effective with the highest concentration resulted in the least growth of mycelium.

Compared with *R. solani* and *S. homoeocarpa*, *Pythium aphanidermatum* was the least sensitive fungus to both bialaphos and PPT. At least 500 mg/l of bialaphos supplement was needed to significantly reduce the mycelial growth on the PDA medium) and the whole plate was covered with the mycelium of *Pythium aphanidermatum* one week after the initial inoculation. The presence of PPT had no effect on the inhibition of *Pythium aphanidermatum* up to the highest concentration (600 mg/l) amended in PDA medium. However, the amendment of bialaphos and PPT did show some inhibitory effect on the growth of *Pythium aphanidermatum* when the amount of mycelium, instead of the measurement of radial length of mycelium, was employed as the indicator to represent the growth of *Pythium aphanidermatum*..

B. Greenhouse test of transgenic creeping bentgrass for suppression of pathogenic diseases

Various concentrations of bialaphos solution were applied on transgenic creeping bentgrasses expressing the bialaphose-herbicide resistance *(bar* ) gene to assess the effects of bialaphos spraying on the development of the three different pathogens. The application of bialaphos had a very significant effect on the suppression of brown patch disease development when the disease rating was taken one week after the fungus inoculation. When bialaphos application was executed three hours before the inoculation of *Rhizoctonia solani* on transgenic plants, disease symptoms were rarely observed, and there was only minimal plant damage due to the infection of *Rhizoctonia solani*. At the concentration of 200 mg/l of bialaphos solution, about one-tenth of the recommended herbicide spraying rate to kill untransformed creeping bentgrasses, the application significantly reduced the plant damage due to pathogen infection.

Transgenic plants that were not treated with bialaphos showed typical symptoms of brown patch disease and a significant amount of plant damage. Even two days after the pathogen inoculation, when the disease began to develop, the application of bialaphos could still significantly restrain the growth of mycelium and the development of brown patch disease. The untreated control plants, either transgenic or nontransgenic, were severely damaged by the infection of *R. solani*. The grass blades became water soaked and dark at first but soon became dry, wither, and turned light brown. The disease was able to continuously develop even after plastic bags were taken off and a lot of the untreated plants were dead three weeks after the pathogen inoculation (data

not shown). There was no significant difference observed between two different timings of bialaphos application ($F = 0.29 < F_{.05}(1, 63) = 4.00$).

The bialaphos application, either three hours before or two days after the pathogen inoculation, was also very effective in preventing the disease development of *S. homoeocarpa*, the etiologic agent of dollar spot, on transgenic creeping bentgrasses. The plant damages on transgenic bialaphos-resistant creeping bentgrasses after the bialaphos application were significantly less than those on transgenic plants not treated with bialaphos. The difference between the two application times was significant ($F = 8.23 > F_{.05}(1, 81) = 3.98$), and there was more plant damage caused by the infection of *S. homoeocarpa* when the bialaphos spraying on transgenic plants was done two days after the pathogen inoculation.

The development of disease symptoms of dollar spot on transgenic and nontransgenic control plants which were not applied with any bialaphos solution was not as rapid and severe as that of brown patch, and plant damage of untreated plants caused by the fungal infection was also less severe than that of *R. solani* in our testing system. Most untreated control plants showed small, circular, sunken white patches when covered with plastic bags and a few of them were able to recover from the damage caused by the infection of *S. homoeocarpa* when plastic bags were taken off two week after the data of disease rating had been collected.

The bialaphos application was more effective in protecting against the infection of *R. solani* than against the infection of *S. homoeocarpa*. There were more plant damages observed on transgenic bialaphos-resistant creeping bentgrasses due to the disease development of the dollar spot pathogen than those of the brown patch pathogen after the application of bialaphos. However, the disease development was significantly restrained and most plants were able to completely recover from the infection and grew normally.

Though the spraying of bialaphos, applied either three hours before or two days after the pathogen inoculation, was effective in the prevention of disease development as reflected by the results that the increases in the concentration of bialaphos did reduce the plant damage caused by either brown patch or dollar spot, the treatment means were not significantly different. The lowest applied rate of 200 mg/l of bialaphos was high enough to suppress the disease development of both fungal pathogens. The results also showed that a single application of bialaphos could suppress the disease development of *Pythium* blight, though not as effectively as with brown patch and dollar spot. When 200 mg/l of bialaphos was applied three hours before the pathogen inoculation, it significantly restrained the infection of *Pythium aphanidermatum* and reduced the amount of plant damage one week after the initial inoculation. Better disease control was achieved when higher rate of bialaphos spraying was applied on transgenic plants. Higher concentration of bialaphos (at least 800 mg/l) was needed to significantly reduce the plant damage due to the infection of *Pythium* blight when bialaphos was applied two days after the inoculation. bialaphos application on transgenic plants before the pathogen inoculation provided a little better protection against the infection by *Pythium aphanidermatum* ($F = 25.57 > F_{.05}(1, 153) = 3.96$). However, no matter which application time and concentration of bialaphos were employed in this study, the infection of *Pythium* blight was severe and caused more plant damage than the other tested pathogens when disease symptoms

were examined two weeks after the initial inoculation (data not shown).

C. Discussion on control of pathogenic diseases in transgenic herbicide resistant creeping bentgrass

bialaphos exhibited inhibitory activity *in vitro* against the growth of *R. solani*, *S. homoeocarpa*, and *Pythium aphanidermatum* that was superior to PPT, as reflected by their ED50 values. Increasing concentrations of bialaphos and PPT were of greater effectiveness, with the highest rates resulting in the smallest colonies of mycelium. When working with mean values of ED50 for *R. solani* and *S. homoeocarpa*, the ED50 values for PPT were higher than those for bialaphos. It is surprising that the mycelial growth of *R. solani* was significantly inhibited at the concentration of one mg/l bialaphos (21 % reduction).

The same trend was also evident in the case of *Pythium aphanidermatum*, though the inhibition of the mycelial growth due to the inclusion of bialaphos in PDA medium was not as significant as with *R. solani* and *S. homoeocarpa*. There was no significant inhibition of the mycelium of *Pythium aphanidermatum* detected up to the highest concentration of supplement (600 mg/l) in our treatment regimes of PPT when radial length was used to measure the growth of mycelium. However, both bialaphos and PPT did show certain degree of inhibitory effect on the amount of mycelial growth of *Pythium aphanidermatum*. The inhibition by bialaphos of growth of *Pythium aphanidermatum* was corroborated by the protection that the application of bialaphos on transgenic plants provided against the infection by *Pythium aphanidermatum* and by the reduction on the plant damage due to *Pythium* blight. It is intriguing to observe that different fungal pathogens showed the same trend in differential *in vitro* responses toward bialaphos and PPT. bialaphos is a tripeptide precursor of PPT, an analogue of glutamate, in which two alanine residues are linked to the PPT. While PPT is an inhibitor of glutamine synthetase in both plant and bacteria, the intact tripeptide has little or no inhibitory activity *in vitro* 5,7. In both bacteria and plants, intracellular peptidases remove the alanine residues and release active PPT (*L*-phosphinothricin).

Though the PPT used in this study was a mixture of *D*- and *L*-phosphinothricin (ammonium-*DL*-homoalanin-4-ylmethylphosphinat), in which the *D*-isomer is the inactive inhibitor of glutamine synthetase and the *L*-isomer is the active moiety of tripeptide bialaphos, it is still difficult to explain the significant differences shown in the magnitude of ED50 values where the sensitivities of *R. solani* and *S. homoeocarpa* for bialaphos were higher than those for PPT. Though it has not been reported, we speculated, however, that the *D*-isomer, the inactive inhibitor of glutamine synthetase, might have interfered with the *L*-isomer, the active moiety of tripeptide bialaphos, in the binding of glutamine synthetase and reduced the inhibition efficiency of *L*-PPT. It is even more surprising to know that the bialaphos provided a better selection efficiency in killing nontransgenic callus of creeping bentgrass than the PPT did (manuscript in preparation). Though it has been remarked that the inefficiency of PPT selection was due to the interference of glutamine or asparagine in the culture medium with herbicide activity22, it seems to us that it is more or less due to the nature of PPT. The composition of selection media used to establish the kill curves for bialaphos and PPT were identical except for the selective agent in our experiment. Compared to PPT, bialaphos was able to kill nontransgenic callus more effectively, at a lower concentration, and within a shorter period of selection. It would be interesting to see whether the same trend could be applicable to other plant materials

and fungus species.

Certainly, the relative sensitivity to bialaphos and PPT was significant, but the basic difference in the values of ED50 for the three different fungi was significant as well. In our *in vitro* test, the three pathogens also showed different responses to bialaphos and PPT. *R. solani* was most sensitive to the presence of either bialaphos or PPT and *Pythium aphanidermatum* was the least. It has been noticed for some time that the application of one of several fungicides, such as Benomyl, Chlorothymol, Cycloheximide+Thiram, PCNB, and Triadimefon, could provide an efficient control of brown patch and dollar spot diseases at the same time. However, they could not effectively control *Pythium* blight in most cases. Most the fungicides designated to control *Pythium* blight, such as Chloroneb, Ethazole, Metalaxyl, and Propamocarb, were not able to prevent infection by either *R. solani* or *S. homoeocarpa* 1.

The *in vitro* sensitivity data help explain some of the efficacy trends observed in the greenhouse study. Application of bialaphos on transgenic bialaphos-resistant creeping bentgrasses, even at the lowest rates, showed universal effects in suppressing disease development and reducing plant damage due to fungal infection. Bialaphos spraying was most significant in restraining the disease symptoms of *R. solani*. Both spraying times showed significant effectiveness in the suppression of the disease development of brown patch.

*S. homoeocarpa* was also significantly sensitive to the *in vivo* application of bialaphos; however, the application before the pathogen inoculation provided a better control of dollar spot. But even if bialaphos was applied two days after the pathogen inoculation when the pathogen had started to develop and spread, it still provided good plant protection and was able to significantly restrain the disease symptoms of *S. homoeocarpa*.

Though it was not as effective as in the cases of *R. solani* and *S. homoeocarpa*, bialaphos spraying still limited plant damage due to infection by *Pythium aphanidermatum*. The timing of bialaphos application was also important in suppressing the disease symptoms of *Pythium* blight. Better results in reducing plant damage were obtained when bialaphos was applied three hours before the pathogen inoculation.

Interactions between herbicides and plant pathogens have been well documented[8; [9]. The main cause of this phenomenon is that the biological activity of pesticides may extend beyond its effects on the target organisms. Upon treatment with herbicides, plant diseases caused by fungal pathogens have been reported to increase [10; 11] or decrease[12] resistance to diseases. More research needs to be done not only to assess the applicability of the antifungal activity of bialaphos toward other fungi, but also to investigate the mechanism of its inhibitory effect so that we might better understand the interactions between bialaphos and fungal pathogens and explain the differing reactions of the various fungi to the application of bialaphos.

Bialaphos has mainly been used as a broad-spectrum contact herbicide and as a selective agent in plant transformation experiments. However, it has been reported that it could be used as an effective selective agent to improve the transformation frequencies of *Cercospora kikuchii*, a fungal pathogen of soybean. Their report and the results of our *in vitro* study, where the bialaphos showed significant inhibitory

effects toward *R. solani* and *S. homoeocarpa*, suggest that bialaphos could be used as an efficient fungicide for a variety of fungal pathogens.

In our studies on creeping bentgrass, the application of 200 mg/l of bialaphos, which is about one tenth of the recommended concentration to kill untransformed turfgrass plants, was enough to significantly reduce plant damage due to the infection of both *R. solani* and *S. homoeocarpa*. The low rates at which bialaphos was effective present a novel and economical means for the control of some fungal pathogens. These facts coupled with the results presented in this report which show that the application of bialaphos could prevent or suppress the infection by several fungal diseases indicate that it may, therefore, be possible to combat fungal infections and weed infestation simultaneously in fields of bialaphos-resistant creeping bentgrasses by a judicious choice of concentration, frequency, and time of application.

## V. Anticipated Results for the Coming Year

Thousands (many lines) of transgenic creeping bentgrass containing the herbicide resistance gene have been developed. These plants, after being sprayed with Liberty herbicide, are resistant to dollar spot and brown patch diseases. Also, over thousand (many lines) of transgenic creeping bentgrass containing the potato proteinase inhibitor II gene have been developed. Furthermore, hundreds (several lines) of transgenic creeping bentgrass containing the drought resistance gene (mtlD gene) were developed. These plants have been co-transformed with chitinase gene. We hope that more funds will be provided to PIs to confirm the integration and expression of the chitinase gene in these plants, test these plants for their resistance to turfgrass diseases, and field test them during summer of 1997.

## VI. Generated publications


1. M. Sticklen, D. Warkentin, C-A Liu, R. K. Hajela, L. Graham, H. Zhong, B. Peterson, J. Vargas, and B. Branham (1995). Genetic engineering in *Agrostis palustris* Huds. (creeping bentgrass). In: Y. P. S. Bajaj (ed.). Biotechnology in agriculture and forestry. Plant protoplasts and genetic engineering of plants. Vol. 38 . **Springer Verlag, Publs.** Berlin, Germany. pp. 151-162.

2. Chien-An Liu (1995). Engineering herbicide resistance in creeping bentgrass and its potential application on the prevention of fungal diseases. **Ph. D. Thesis.** Michigan State University.

3. Chien-An Liu, Joseph Vargas, and Mariam Sticklen (1996). Genetic engineering of creeping bentgrass with a protease inhibitor II gene. Ready for submission in **Plant Cell Report.**

4. Chien-An Liu, Joseph Vargas, and Mariam Sticklen (1996). Simultaneous control of weeds, dollar spot and brown patch diseases in transgenic herbicide resistance creeping bentgrass. Submitted to **Weed Science.**

**VII. References Used in this Report**


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[12] Grinstein, A., Lisker, N., Katan, J., and Eshel, Y. 1984. Herbicide-induced resistance to plant wilt diseases. Physiol. Plant Pathol. 24: 347-356.