The Significance of Plant Wounds in Effective Control of Annual Bluegrass (Poa annua L.) with Xanthomonas campestris pv. poae (JT-P482)*

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Introduction
Annual bluegrass (Poa annua L.) is one of the most persistent weeds growing in golf course greens, and the use of chemical herbicides to eradicate it carries the risk of damage to desirable turf grasses such as creeping bentgrass (Agrostis palustris Huds.) or Kentucky bluegrass (Poa pratensis L.)

In order to resolve this problem, research was initiated into bioherbicides which utilize the specificity of plant pathogens and remain highly selective and safe to the environment. The phytopathogen Xanthomonas campestris pv. poae was isolated and the isolate JT-P482 was selected as a candidate for development in 1993. Subsequently, efficacy tests both in the greenhouse and in the field were carried out. Tests checking the safety of the isolate to plants, animals and the environment, and several toxicity tests were successfully completed. In May, 1997, registration of this bacterium as the bioherbicide "CAMPERICO" was approved in Japan.

The results of a large amount of research showed that the most crucial factors affecting the efficacy of the isolate were temperature conditions and the cell concentration of the inoculum. In general, for enhancement of efficacy, the formulation and application techniques are also important. However, because this is a bacterial herbicide, the technique by which injury is administered to the plant, allowing the bacteria a means of entry, is additionally of great significance.

In this report, greenhouse experiments carried out to determine the effects of different injuring techniques on the control of annual bluegrass are described. The experiments concern three main areas: (1) the effect caused by injury to different parts of the plant, and that caused by different numbers of injury; (2) the effect caused by the time interval between injury and treatment, and that resulting from whether injury is carried out before treatment or vice versa; and (3) the effect of continual mowing on the population of viable bacteria within annual bluegrass.

Materials and Methods

General
Approximately 25 mg of annual bluegrass seeds were sown in 5 \times 5 \times 5\text{cm} deep plastic pots filled with sand. These pots were placed in the greenhouse (20°C / 15°C, day / night temperature) for 4 weeks, by which time each pot contained approximately 40 seedlings possessing 4-5 leaves each. JT-P482 (2 \times 10^9 colony forming units = CFU/ml) in 10% glycerol stored at -80°C was thawed with distilled water. The suspension was adjusted to an

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optical density of 0.1 absorbance at 575nm, containing $10^4$ CFU/ml to obtain the final concentration of $10^5$ CFU/ml, as previously reported). Accurate determination of the number of viable cells in a bacterial suspension was made by the dilution-plate counting method.

(1) The effect caused by injury to different parts of the plant, and that caused by different numbers of injury.

Taking into account the various ways in which annual bluegrass can be cut by the blade of a mower used for golf greens, the experimental blocks were laid out and cut and treated as follows (an illustration of the plant parts referred to is shown in Fig.1):

Fig. 1 Illustration of the different sites at which injury was administered to annual bluegrass plants prior to the application of JT-P482.

1. Clipping of the whole plant at a height of 5cm [cut all]; 2. Clipping of one of the most developed leaves [leaf-1]; 3. Clipping of three of the most developed leaves [leaf-3]; 4. Clipping of five of the most developed leaves [leaf-5]; 5. Clipping of the youngest leaf [young-1]; 6. Clipping of the lowest leaf [old-1]; 7. Clipping of one stem (at a height of 5cm) [stem-1]; 8. Clipping of the stem just below the seedhead [seedhead]; 9. Abrasion of one of the most developed leaves with sandpaper [rub-1]; 10. Abrasion of five of the most developed leaves with sandpaper [rub-5]; 11. Cutting of one portion of root at a 3cm depth from the sand surface with a knife [root-1]; and 12. Cutting of five portions of root at a 3cm depth from the sand surface with a knife [root-5].

In the case of blocks 1 to 8, clipping inoculations were carried out with scissors dipped in JT-P482 suspension ($10^6$CFU/ml). In the case of blocks 9 to 12, a JT-P482 suspension ($10^6$CFU/ml, 400ml/m² water volume) was sprayed with an airbrush to previously injured plants as described above. A check control for each experimental block was cut and treated in the same way with sterile distilled water. In addition, un-clipped annual bluegrass was sprayed with the JT-P482 suspension, as described above, as a positive check control block. Treated annual bluegrass and check plants were placed in the greenhouse (25°C/20°C, day/night temperature) and disease development was observed. Evaluation was carried out every week starting 9 days after treatment (=DAT) until 37 DAT, based on visual estimation from 0% = healthy to 100% = death. Six replications were made of all experimental blocks.

(2) The effect caused by the time interval between injury and treatment, and that resulting from whether injury is carried out before treatment or vice versa.

Annual bluegrass plants grown as described above were clipped to a height of 5cm every day for 7 days with a sterile electric clipping machine, simulating the conditions experienced by plants mown in greens, then the plants were used for the experiments. The inoculum was prepared as described above. The intervals between injury and inoculation or between inoculation and injury were 0, 30 min. and 1, 2, 3 and 6 h. Each treatment block was separated into two, in one of which the injury preceded the application of inoculum (cut & spray method), and in the other of which inoculum was applied prior to injury (spray & cut method). In each case, cutting was
carried out at a height of 2 cm using sterile scissors and JT-P482 (10^1 CFU/ml, 400 ml/m² water volume) was sprayed with an airbrush. This experiment was carried out in temperatures of 25°C/20°C, in three humidity conditions as follows: (a) normal humidity (35-50% R.H.) in the greenhouse, (b) dry conditions produced by an electric fan at a wind velocity of 4.6 m/s and (c) high humidity (100% R.H.) produced by sealing pots in plastic bags containing a little water. Check treatments for each experimental block were carried out using sterile distilled water. After these treatments, all annual bluegrass plants were placed in normal humidity conditions (a), and disease development was observed. All experiments were conducted with 6 replications. Evaluation is presented as % control from measurement of the fresh weight (=FW) of top growth 21 DAT.

Control (%) = (FW of check plant - FW of treated plant)/FW of check plant x 100

(3) The effect of continual mowing on the population of viable bacteria within annual bluegrass.

Bearing in mind that putting greens are mown on a daily basis in season, the effect of this on bacterial populations in annual bluegrass after treatment was investigated. A rifampin-resistant mutant of JT-P482, designated as Rif-482, was used to measure bacterial populations over time. The isolation of Rif-482 and its screening for similarity to the wild type JT-P482 have been previously described 6). The technique for detection of bacterial populations in whole plants is as follows: fine cut plant tissue was spread on YNagar (yeast extract 5 g/L and nutrient agar 23 g/L) containing rifampin (30 μg/ml). Tenfold serial dilutions of the sample suspensions were made. The inoculum concentration used was 10^1 CFU/ml. Clipping inoculation was carried out to annual bluegrass at a height of 1.5 cm from the surface of the sand and a vacuum cleaner was employed to ensure that inoculum did not adhere to any parts of the plant except the clipped surface. Then, inoculated plants were moved to the greenhouse (25°C/20°C) and bacterial populations were counted immediately (about 30 min.) after treatment, and 1, 3, 7 and 14 DAT. Plants were clipped every day to a height of 1.5 cm and on evaluation days individual representative plants were examined for viable bacterial numbers. Viable bacterial numbers in plants were measured separately in the divided parts of the 1.5 cm length of stem (= cutting height), the upper 7.5 mm and the lower 7.5 mm (Fig. 4), and in 0.5 g of roots washed with tap water as previously reported 6).

Results and Discussion

(1) The effect caused by injury to different parts of the plant, and that caused by different numbers of injury.

A comparison of disease development in each treatment showed that the time taken to achieve 80% control differed according to each inoculation method (Fig. 2).

Fig. 2 Effect of JT-P482 treatments on annual bluegrass plants injured by the different methods illustrated in Fig. 1. ① clipping of the whole plant at a height of 5 cm, ② clipping of one of the most developed leaves, ③ clipping of three of the most developed leaves, ④ clipping of five of the most developed leaves, ⑤ clipping of the youngest leaf, ⑥ clipping of the lowest leaf, ⑦ clipping of one stem (at a height of 5 cm), ⑧
clipping of the stem just below the seedhead, abrasion of one of the most developed leaves with sandpaper, abrasion of five of the most developed leaves with sandpaper, cutting of one portion of root at a 3 cm depth from the sand surface with a knife, and cutting of five portions of root at a 3 cm depth from the sand surface with a knife.

Control(%) = (FW of check plant – FW of treated plant)/FW of check plant × 100

Clipping of whole plants [cut all] took the shortest time: 8 days. Clipping of one stem [stem-1] took 13 days to reach 80% control. Clipping below the seedhead [seedhead] took 28 days. Clipping of leaves showed that five leaves [leaf-5] needed 22 days, three leaves [leaf-3], 24 days, and one leaf [leaf-1], 31 days. The slowest disease development showed in the clipping of the lowest leaf [old-1] which had not achieved 80% control by the end of the experiment (37 days). Abrasion treatments of the leaves [rub-1 and rub-5] and cutting treatments of roots [root-1 and root-5] demonstrated intermediate control rates. While degeneration, like chlorosis, was observed in the case of spray inoculation to un-clipped annual bluegrass plants, no disease (e.g. wilting or desiccation) was observed before the end of the experiment (data not presented).

As indicated above, a larger number of injuries resulted in greater effectiveness. This is shown by a comparison of the results from stem clipping methods and leaf clipping methods, and from leaf clipping methods. Also, disease development was more rapid when injuries were made to stems rather than leaves. This may be due to the difference in the width of the xylem areas exposed by clipping, as the area of the clipped surface of a stem is larger than that of a leaf. Similarly, it has been reported that the length and diameter of the xylem in elms are factors in the tree’s resistance to a fungus.

Also, it may be that inoculation carried out to the main organ of translocation (=stem) assists the spread of systemic infection along with water-translocation. The clipping treatment of the stem just under the seedhead showed a longer time before disease appearance compared with the clipping treatment of the stem closer to the basal parts of the plant. Furthermore, in the oldest leaf treatments, though plant desiccation occurred so did repeated regrowth, and slower disease development was observed than in other leaf treatments. It has been determined that in X. campestris pv. graminis, the closer to the stem that the pathogens are inoculated, the faster the disease develops. This might be caused by the shorter distance that the bacteria have to travel in the plant in order to cause a systemic infection.

This leads to the conclusion that to obtain maximum effectiveness, it is important that the leaves and particularly the stems of annual bluegrass in putting greens are in an upright position in order for the maximum number of wounds to be made by mowers. Therefore, thatching or grooming treatments prior to mowing just before JT-P482 application would have a positive influence on effectiveness.

In the case of the abrasion treatments, which simulate the blades of the mower touching only the surface of the leaf, (a possible occurrence because of the horizontal, creeping growth pattern of the weed in turf) it appears that invading bacterial numbers in plants are lower than those in the clipping treatments.

In the case of the plant inoculated to its lowest, oldest leaf, which showed the slowest disease development, plant desiccation and repeated regrowth occurred, but no complete plant death was observed.

Though it is known that JT-P482 does not survive for very long in soil, when the suspension comes into contact with injured roots, due to turf management practices for
example, then the pathogen can spread to the rest of the plant and infection can be established (11), (12).

It can be seen that this pathogen has the potential to systemically infect annual bluegrass regardless of how the necessary wounds are administered, since, with the exception of the lowest leaf, even inoculation to only one leaf caused plant death. But to obtain further enhancement of the efficacy, it is recommended that large areas of the plant should come into contact with JT-P482 suspensions, or that wounds should be administered closer to basal parts of plants.

(2) The effect caused by the time interval between injury and treatment, and that resulting from whether injury is carried out before treatment or vice versa.

In the investigation into the effects of the interval between injury and inoculation, two methods of treatment (cut and spray; spray and cut) in three different conditions of humidity were studied (Fig. 3). In the cut & spray experiment, no significant differences in effects were observed between plants in the different humidity conditions (35-50% R. H., drying by electric fan and 100% R. H.) regardless of the different time intervals between cutting and spraying of up to 6h. However, the spray & cut experiment showed a marked efficacy loss under dry conditions when cutting was carried out 2 or 3h after spraying. All these results were obtained under relatively high temperature conditions (25°C/20°C) and it is possible therefore that higher efficacy was obtained than may be the case under more severe circumstances such as lower temperatures or more arid conditions. A previous study showed significant efficacy reduction in a pot experiment set in the field where inoculation was performed 1h after mowing (unpublished data), though weather conditions were different. A comparison of the results from that experiment and this one showed inconsistency, but for efficient use, it seems that the interval between cutting and JT-P482 treatment should be as short as possible. In addition, if treatment is carried out in severe conditions (e.g. dryness), the cut & spray method is recommendable for practical use.

**Fig. 3** Effect of interval between injury and JT-P482 inoculation in different humidity conditions. Experiments were carried out under 25°C/20°C temperature conditions. The humidity conditions are described in the box. “Fan” means dry conditions produced by an electric fan at a wind velocity of 4.6 m/s. %Control = (FW of check plant − FW of treated plant) FW of check plant × 100. Error bar is equal to standard deviation.

**Fig. 4** Illustration of the parts of annual bluegrass used in the experiment to ascertain the effect of continual mowing on populations of viable bacteria. Results are presented in Fig. 5.
(3) The effect of continual mowing on the populations of viable bacteria within annual bluegrass.

Turf management generally requires the removal of clippings from mowing and therefore the removal of the bacteria multiplying in the clippings. As a result, the change in the viable numbers of bacteria remaining in plants which had been subjected to continual mowing on a daily basis was investigated. Immediately after treatment, bacterial numbers were $5.3 \times 10^7$ CFU/g fresh weight (= FW) in the upper part of the stem sampled, $2.9 \times 10^7$ CFU/g FW in the lower part, and were undetectable in the root part sampled (Fig. 5).

Seven days after treatment the bacterial numbers in the upper, lower and root parts were $1.8 \times 10^9$, $2.5 \times 10^9$ and $5.4 \times 10^9$ CFU/g FW respectively, indicating that the bacteria had multiplied sufficiently to have the potential to cause the death of plants$^6)$. 14 days after treatment, all annual bluegrass plants were dead.

Although repeated clipping to a height of 1.5 cm inevitably meant the removal of some of the inoculated bacteria with the grass clippings, it was clarified that the bacteria immediately translocated within the body of the plant after inoculation, then multiplied and reached maximum populations of $10^9$ CFU/g FW (a sufficient number to cause wilting and plant death) 7 days after treatment despite daily clipping.

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Summary

In the use of the phytopathogenic bacteria Xanthomonas campestris pv. poae (JT-P482) as a bioherbicide to control annual bluegrass, a wound is necessary for the bacteria to enter the plant. In this paper, the relationship between wound treatments and efficacy is described. (1) The more wounds, especially in parts of the plant closer to the stem, the greater the efficacy obtained. Therefore, in putting greens, it is important to make annual bluegrass stand upright by using a thatching-reel mower or grooming techniques before mowing. (2) For efficient use, the interval between cutting and JT-P482 treatment should be as short as possible. In addition, if treatment is carried out under severe conditions (e.g. dryness) the cut & spray method is recommendable for practical use. (3) Changes in the bacterial numbers in annual bluegrass plants after repeated mowing (to a height of 1.5 cm) were determined. Seven days after treatment, bacterial populations in plant tissue reached a maximum ($10^9$ CFU/g FW)
and this was sufficient to cause plant wilting and death.

References


植物病原細菌 *Xanthomonas campestris pv. poae*
（JT-P482）によるスズメノカタピラの防除
－付傷が効果におよぼす影響－

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要約

スズメノカタピラに特異的な植物病原細菌*Xanthomonas campestris pv. poae*（JT-P482）による微生物除草剤を開発し、1997年「キャンペリコ」名で農薬登録を取得した。本施用法に最も特徴的である「付傷」に関し、温室内で試験を行った結果について報告する。

(1) 付傷箇所数が多いほど効果は速くあらわれた。葉に対する付傷より茎に対する付傷のほうが速く効果が表れた。グリーン内でより効果をあげるためには、スズメノカタピラの茎や葉がより効率的にモアーの刃で刈られるように直立していることが望まれる。

(2) 菌の噴霧の前または後に付傷を行い、それぞれ付傷と処理の間隔が効果におよぼす影響をみたところ、付傷後処理までの時間は6時間であっても効果には大きな影響を与えなかった。しかし噴霧処理後の付傷では、風量の多い場合には2、3時間で著しい効果の減少が認められた。

(3) 処理後、連日切り込みを行ったスズメノカタピラ体内の発菌数の変化を調査した。7日後に10⁷CFU/g FWと、植物を枯死させるに十分な菌数にまで増殖したことが判明した。

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