Studies on the Chemical Control of Reproductive Functions in Higher Plants

- Physiological and Biochemical Characteristics of Rice Sterility Induced by Chemical Treatment -

高等植物における生殖機能の化学的制御に関する研究

－ 化学的処理により誘導される稲不稔の生理、生化学的性質－

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Introduction

The improvement of crop plants through the production of hybrid varieties is a major goal of plant breeding.[1] Crosses between inbred plant lines often result in progeny with higher yield, increased resistance to disease, and enhanced performance in different environments compared with the parental lines.[2]

The production of hybrid seed on a large scale is challenging because many crops have both male and female reproductive organs (stamen and pistil) on the same plant. However, the large-scale directed crosses between inbred lines makes difficult to accomplish. To guarantee that outcrossing will occur to produce hybrid seed, breeders have either manually or mechanically removed stamens from one parental line, used natural self-incompatibility systems that prevent self-pollination, or exploited male sterility mutations that disrupt pollen development.[3,4] Manual emasculation is labour intensive and impractical for plants with small bisexual flower (such as rice, wheat), many crop plants do not have self-incompatibility or male sterility genes, and use of male sterility requires a fertility restorer system.[2,5]

The chemical induction of male sterility in rice (*Oryza sativa* L., Japonica) is potentially atractive, because the utilization of heterosis for rice breeding is limited. The limiting factor in breeding is the prolonged process of developing male-sterile and restore lines for hybridization. This disadvantage could be overcome if chemicals were used to induce male sterility.[2,6-8] Consequently, chemicals may be used as breeding tools and as a means to produce hybrid seeds if they induce complete male sterility without causing female sterility and

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crop injury. Furthermore, they must be unaffected by environmental variability such as rain, wind, and temperature during crop growth stage and be suitable for various cultivars.[6-8]

Although 2-chloroethylphosphonic acid (ethrel),[9] sodium 1-(p-chlorophenyl)-1,2-dihydro-4,6-dimethyl-2-oxonicotinate(RH-531)[10,11] and disodiummethanearsonate (DSMA)[8] are known to induce male sterility in rice, these chemicals have several problems associated with their use such as high application dose (ethrel; 55 mM, RH-531; 0.3 mM, and DSMA; 1 mM), a propensity to cause crop injury and a failure to bring about complete male sterility (Fig. 1).
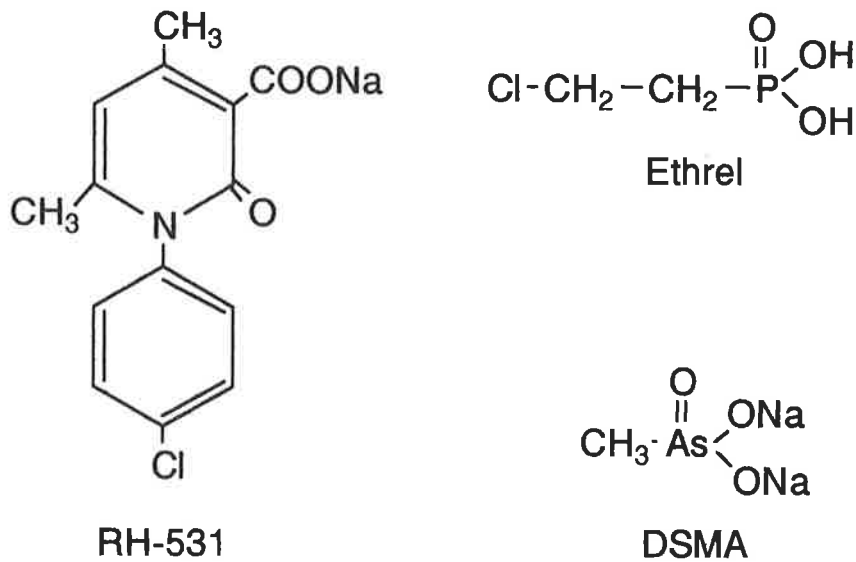
Fig. 1 The Structures of Male Sterilants for Rice Plants

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For such reasons, they do not have any practical use. In order to develope effective chemicals to induce male sterility in rice, it is necessary to study their mode of action. For example, DSMA reduces the activities of succinic dehydrogenase and cytochrome oxidase, two respiratory enzymes; consequently, it suppresses the normal growth of pollen.[8] However, little study has been devoted to the mode of action of chemicals on male sterility.[2,8]

Furthermore, screening for new rice sterilants among microbial metabolites is the important program because the development of the chemicals by artificial synthesis is limited, and natural products[12-26] provide with new structural classes which can serve as models for the syntheses of the effective rice sterilants. From this point of view, S35-C2, a metabolite from actinomyces, was found as an effective rice sterilant. Moreover, kasugamycin,[27,28] aminooxyacetic acid (AOA)[29-39] and bialaphos[40-46] were found as the effective rice sterilants. Bialaphos especially induces complete sterility in rice at a very low concentration, and bialaphos is the most effective sterilant of the known chemicals used for inducing sterility.

In this study, the author found the new effective rice sterilants and investigated the physiological and biochemical characteristics of rice sterility induced by chemical treatment.

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Materials and Methods

Assay procedures to rice plants

Materials. Kasugamycin hydrochloride, aminooxyacetic acid (AOA), gibberelline (GA$_3$), α-naphthylacetic acid (NAA), benzotriazole, D-cycloserine, hydroxyproline, α,α-dipyridil, benzyladenine (BA), kanamycin sulfate, tetracycline hydrochloride, rifampicin, guanidine thiocyanate, 1-(2-tetrahydrofuryl)-5-fluorouracil, L-glutamine, ammonium sulfate, and ammonia solution were purchased from Wako Pure Chemical Co.. 1-Aminocyclopropane-1-carboxylic acid (ACC), spermine tetrahydrochloride, methylglyoxal bis-(guanylhydrazone) dihydrochloride (MGBG), L-azetidine-2-carboxylic acid (ACA), 2-chloroethylphosphonic acid (Ethrel), erythromycin, spectinomycin dihydrochloride, and L-methionine sulfoximine (L-MSO) were purchased from Sigma Chemical Co.. Bialaphos and L-phosphinothricin (L-PTC) was kindly provided by Meiji Seika Co..

Assay procedures. Rice plants (*Oryza sativa* L., cv. Yamabiko) were germinated and planted in paddy soil. At the two- or three-leaf stage, three weeks after the germination, the seedlings were transplanted into 2 kg of paddy soil in 16-cm diameter pots. Each pot held one seedling and was fertilized with 120 ppm N and 180 ppm P as diammonium hydrogenphosphate, and with 140 ppm K as potassium chloride. The rice plants were grown under natural sunlight at a temperature from 20 to 35 °C. The developmental stages in rice were ascertained by measuring the length of the young panicle, and the distance between auricles of the

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last two leaves in the main tiller.[47)] The developmental stages are defined as the panicle formation stage when the young panicle reached 0.4 - 0.5 mm in length, and as the reduction division stage when this distance was -4 to +4 cm.

The chemicals to be tested were formulated as an aqueous solution containing 0.1 % Tween-80 as a wetting agent and 5 % acetone to aid solubility. The aqueous solution of each chemical was adjusted to pH 7.0 with 1N NaOH and sprayed on all leaves of the rice plant with an atomizer at the rate of 10 ml per pot. Three replications were used for each treatment.

At ear emergence, all the heads from each pot were placed in glassine bags to prevent cross pollination. After the bagged ears had been harvested at maturity, the seed set, spike length, culm length and weight of the ripe seeds were recorded and compared with the values for an untreated control.

Physiological and biochemical assay procedures to rice sterility

Assay for glutamine synthetase activity in panicles. Panicles (1 g) were washed and cut into 5 mm length. The sample was homogenized with a Polytron (Kinematica Co.) at the medium speed setting for 60 sec at 0 °C. The homoginizing medium was composed of imidazole-HCl buffer (pH7.2; 50 mM), EDTA (pH7.2; 0.5 mM) and 2-mercaptoethanol (5 mM). After the extract was filtered through four layers of cheesecloth, the filtrate was centrifuged at 15,000xg for 15 min and the supernatant was used for the assay of GS activity.

Glutamine synthetase (GS) activity was measured by the formation of γ-glutamylhydroxamate in the sythetase reaction.[44-54] The reaction mixtures contained enzyme (100 μl), sodium L-glutamate (50 μM), NH$_2$OH (pH7.2; 100 μM), NaATP (10 μM), MgCl$_2$ (20 μM), imidazole-HCl buffer (pH7.2; 50 μM), 2-mercaptoethanol (25 μM), in a final volume of 1.0 ml. After incubation at 37 °C for 15 min, the reaction mixture was treated with 1.5 ml of ferric chloride reagent contained ferric chloride (0.37 M), HCl (0.67 M) and TCA (0.20 M). The formation of γ-glutamylhydroxamate was measured at 530 nm (Shimazu UV 2200 spectrophotometer). The standard curve was plotted in the manner described above by utilizing 1 ml samples of γ-glutamylhydroxamate (0.1 to 0.7 μmole).

Assay for ATPase activity in panicles. Panicles (1 g) were collected before 2 days from anthesis, and they were washed and cut into 5 mm length. The sample was homogenized with a Polytron (Kinematica Co.) at the medium speed setting for 60 sec at 0 °C. The homoginizing medium consisted of sorbitol (0.25 M), MOPS-KOH (pH 7.0; 50 mM), EDTA (2 mM) neutralized with KOH, 2.5 mM potassium metabisulfite, and 1.5 % soluble PVP (mol wt 24,000). After the extract was filtered through four layers of cheesecloth, the filtrate was centrifuged at 15,000x*g* for 15 min and the supernatant was used for the assay as ATPase activity.

ATPase activities were determined by measuring Pi hydrolyzed from ATP.[55-63] Pi was determined by the Dulley modification[64] of the Fiske and SubbarRow.[65] The reaction mixtures contained enzyme (50 μl), ATP(2Na) (3 μM), MgSO$_4$ (3 μM), HEPES (pH 6.5, 25 μM), KCl (50 μM), Triton X-100 (0.03 %) and inhibitor, in a final volume of 0.25 ml. After the reaction was carried out at 37 °C for 15 min, the reaction was

stopped by the addition of 2.5 ml of ice cold stopping reagent contained ammonium molybdate (0.25 %), sodium dodesilsulfate (1.3 %),and concentrated H_2SO_4 (1.4 %) and placing the tubes on ice. After the solution was added 0.5 ml of the reducing agent contained 1- amino-2-naphtol-4-sulfonic acid (0.1 %), $NaHSO_3$ (5.7 %), $NaSO_3$ (0.2 %), the test tube was thoroughly shaken by hand and incubated for 10 min at room temperature ,and measured at 530 nm (Shimazu UV 2200 spectrophotometer) for determining released inorganic phosphate . The standard curve was plotted in the manner described above by utilizing 250 μl samples of KH_2PO_4 (10 to 50 μg).

Extraction and analysis of free amino acids for steril anthers. For analysis of free amino acids,[45,66] about 800 anthers were collected before 2 or 3 days from anthesis. The sample was ground in mortar with 70 % EtOH, kept for 2 days at room temperature and then centrifuged at 3,000x*g* for 10 min. The precipitate was further extracted once with 70 % EtOH. The two supernatants were combined and concentrated at 35~40 ∘C under reduced pressure. The extract was dissolved in 2 ml of water and washed twice with the same volume of Et_2O. After the water solution was dried up in vacuum, the extract was dissolved in water and the quantity of free amino acids was determined with an amino acid analyzer (Shimazu LC-6A, column; ISC-07/S1504 Na).

Extraction and analysis of free amino acids for leaves and panicles.

For analysis of free amino acids,[45,66] leaves or panicles (1 g) were collected before 2 days from anthesis. They were dried at 80 °C for 3 days. The dried sample (1 g) was ground by a coffee mill. The dried sample (0.1 g) of leaves or panicles and 10 ml of distilled water added

in 15-ml test tube and heated at 100 °C for 1 hr. After the sample solution was cooled in a tapped water bath, the eluate separated by centrifugation (3000xg, 5 min). The eluate was concentrated at 35~40 °C under reduce pressure. The extract was dissolved in water and the quantity of free amino acids was determined with an amino acid analyzer (Shimazu LC-6A, column; ISC-07/S1504 Na).

Ammonia content in panicles. Panicles were collected after 1, 3 and 5 days from chemical treatment. The sample (1 g) was cut 5 mm long and homogenized in 10 ml methanol-chloroform-water (12:5:3, v/v/v) with a Polytron (Kinematica Co.) at the medium speed setting for 60 sec at 0 °C. The nonturbid layers were produced by the addition of 3 ml water per 10 ml homogenate followed by centrifugation (3000xg, 10 min). The upper layer of methanol-water was contained the polar solutes and was taken for determining ammonia content.[67-70] After 5 ml of the sample solution was added in 30-ml tube, the tube was placed on ice and added 4 ml of water and 2 ml of phenol-nitroprusside solution contained phenol (6 %), nitroprusside (0.02 %), phosphate-citrate buffer (0.1 M, pH 12). After the solution mixed thoroughly, 3 ml of the alkaline hydrochlorite solution, contained sodium hypochlorite (0.1 %) and sodium hydroxide (1 M), was added. The solution mixed thoroughly and incubated for 45 min at room temperature, and was measured at 635 nm by Shimazu UV-2200 spectrophotometer. The standard curve was plotted in the manner described above by utilizing 5 ml samples of NH_4Cl (10 to 50 μg).

Total nitrogen in leaves and culms, and panicles. Leaves and culms, and panicles were collected before 2 days from anthesis. They were dried at 80 °C for 3 days. The dried sample (1 g) was ground by a coffee mill. The ground sample (0.1 g) , 2 ml of concentrated H_2SO_4 and a bit of catalyst (Se-$CuSO_4$-K_2SO_4, 1 : 10 : 50 w/w/w) added in 300-ml Kjeldahl flask and heated for about 5 hr using the electric-heated six-flask digestion stands.[71-73] The cooled digest was diluted to volume in a 20-ml messflask. 1 ml of the solution was neutralized (methyl red as an indicator) with 1 N NaOH. The neutralized solution was diluted to volume in a 10-ml messflask, and 0.5 ml of the diluted solution was used for determination of ammonia content by the indophenol method described above.

Protein nitrogen in leaves and culm,s and panicles. The dried sample (0.1 g) of leaves and culms or panicles and 4 ml of distilled water added in 30-ml test tube and heated at 85 °C for 1 hr. After the sample solution was cooled in a tapped water bath, the residue separated by centrifugation (3000xg, 5 min). The residue was used for determining protein nitrogen by the method described in the section of total nitrogen assay.

Free amino acids content in leaves and culms, and panicles. The dried sample (0.1 g) of leaves and culms or panicles and 10 ml of distilled water added in 15-ml test tube and heated at 100 °C for 1 hr. After the sample solution was cooled in a tapped water bath, the eluate separated by centrifugation (3000xg, 5 min). The eluate was diluted to volume in a 20-ml messflask. The reaction mixture contained 2 ml of the diluted

solution and 2 ml of the ninhydrin reagent.[74-76] This reagent was composed of ninhydrin (0.8 g), hydrindantin (0.12 g), methyl Cellosolve (30 ml) and 4 M acetate buffer (pH 5.5, 10 ml). After the reaction was carried out at 100 °C for 15 min in a boiling water bath, the reaction was stopped in a cooled water bath and added 3 ml of 50 % ethanol. After the test tube was thoroughly shaken on a mixer and incubated for 10 min at room temperature, the absorbance was measured at 570 nm by Shimazu UV-2200 spectrophotometer.[74-79] The standard curve was plotted in the manner described above by utilizing 2 ml samples of L-aspartic acid (3 to 30 μg).

Various carbohydrates in leaves and culms, and panicles. Leaves and culms, and panicles were collected before 2 days from anthesis. They were dried at 80 °C for 3 days. The dried sample (1 g) was ground by a coffee mill. For analysis of the reducing sugar, the total sugar, the crude starch and the total carbohydrates in leaves and culms, and panicles, the analytical methods described below were examined.[80]

Reducing sugar. A sample (100 mg) of the ground leaves and culms or panicles was extracted by hot 80 % ehtanol in a boiling water bath. The residue was further extracted twice with 80 % EtOH. The 80 % ethanol eluates were collected and concentrated under reduced pressure. The residue was dissolved in 5 ml of hot water, and added 0.5 ml of 0.3 N $Ba(OH)_2$ and 0.5 ml of 5 % $ZnSO_4$. After the solution was mixed, the solution was centrifuged at 3000xg for 10 min. The supernatant was diluted to volume in a 20-ml messflask. 1 ml of the diluted solution (the sugar solution) was further diluted to volume in a 5-ml messflask.

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Total sugar. After 1 ml of the sugar solution was hydrolyzed by 1 ml of 4 % H_2SO_4 in a boiling water bath for 15 min, the reaction was neutralized (methyl red as an indicator) with 1 N NaOH. The neutralized solution was diluted to volume in a 20-ml messflask, and 1 ml of the diluted solution was further diluted to volume in a 20-ml messflask.

Crude starch. The residue of the leave and culm or panicle, prepared by the extraction of 80 % ethanol, was dried at 80 °C for 5 hr. After dried, the residue added 2 ml of water and heated in a boiling water bath for 10 min. After cooled in a water bath, the mixture added 2 ml of 60 % perchloric acid and heated in a boiling water bath for 15 min. The supernatant of the reaction mixture was separated by centrifugation (3000×g, 10 min). After the rection residue added 4 ml of 30 % perchloric acid and heated in a boiling water bath for 15 min, the supernatant was separated by centrifugation (3000×g, 10 min). The reaction residue was further reacted once by the same method described above, and the supernatant was collected. The supernatants were collected and diluted to volume in a 100-ml messflask. 2 ml of the diluted solution was heated in boiling water for 2 hr. After cooled, the reaction was neutralized (methyl red as an indicator) with 1 N NaOH. The neutralized solution was diluted to volume in a 10-ml messflask.

Total carbohydrates. A sample (100 mg) of the ground leaves and culms or panicles was hydrolyzed by 3 ml of 0.7 N HCl in a boiling water bath for 3 hr. The reaction mixture was centrifuged at 3000 g for 10 min. The supernatant was neutralized (methyl red as an indicator) with 1 N NaOH, and added 0.5 ml of 0.3 N $Ba(OH)_2$ and 0.5 ml of 5 % $ZnSO_4$.

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After the solution was mixed, the solution was centrifuged at 3000 g for 10 min. The supernatant was diluted to volume in a 20-ml messflask. 1 ml of the diluted solution (the sugar solution) was further diluted to volume in a 20-ml messflask.

Determination of the sugar content of the prepared samples. 1 ml of the diluted sample solution of reducing sugar, total sugar, crude starch, or total carbohydrates and 1 ml of a copper reagent were pipeted into a 30-ml test tube and mixed thoroughly. The reaction mixture was heated in a boiling water bath for 15 min and cooled in a cooled water bath. 1 ml of the arsenomolybdate reagent was then added and mixed thoroughly. After 7 ml of water was added and the mixed solution incubated for 20 min at room temperature, the sugar concentration was measured at 500 nm by Shimazu UV-2200 spectrophotometer.[81-85] The standard curve was plotted in the manner described above by utilizing 1 ml samples of D-glucose (10 to 100 μg).

The copper reagent and the arsenomolybdate reagent was prepared by the methods described below.[85] The copper reagent: 24 g of Na_2CO_3 and 12 g of Rochelle salt were dissolved in about 250 ml of water, then the dissolved copper sulfate (40 ml of a 10 % solution) was introduced with stirring, and this was followed by the addition of 16 g of NaHCO3. 180 g of Na2SO4 (anhydrous) was dissolved in about 500 ml of hot water and boiled to expel air. After cooling, the two solution were united and diluted to volume in a 1000-ml messflask. The arsenomolybdate reagent: 25 g of ammonium molybdate and 21 ml of concentrated H_2SO_4 were dissolved in about 500 ml of water. 3 g of $Na_2HAsO_4 \cdot 7H_2O$ was dissolved in 25 ml of water. The two solution were united and diluted to

volume in a 1000-ml messflask. The mixed solution placed in an incubator at 37 °C for 24 hr.

Determination of chlorophyll content in leaves. Leaves were cut 5 mm long and 1 g of them was extracted in 5 ml of 80 % ethanol. Chlorophyll content was determined by measuring at 665 nm by Shimazu UV-2200 spectrophotometer.[86] Another method for determination of chlorophyll content was carried out by the chlorophyll meter (SPAD-502, MINOLTA Co.).

Assay to rice root activity by α-naphthylamine oxidation. Roots were washed, cut 1 to 2 cm long. 2 g of them, 25 ml of α-naphthylamine (40 ppm) and 25 ml of phosphate buffer (pH 7.0; 0.1 M) were added in 100-ml flask and incubated at 25 °C for 6 hr in a water bath. 2 ml of the sample, 10 ml of water, 1 ml of sulfanilic acid (1 %) in acetic acid (20 %), and 1 ml of NaNO2 aqueous solution (100 ppm) were added in 30-ml test tube, and then it was filled up to 20 ml with water. After 30 minutes, α-naphthylamine concentration was measured at 510 nm by Shimazu UV-2200 spectrophotometer.[87-89] The standard curve was plotted in the manner described above by utilizing 2 ml samples of α-naphthylamine (10 to 40 μg).

Isolation, structure analysis, and the physiolosical and biochemical assay procedures for S35-C2

Screening test for GS inhibitor from actinomycetes

Isolation and cultivation of microorganism. Test strains were isolated from soil by conventional methods. About 600 strains of actinomycetes were isolated. These strains were inoculated into test tubes with 5 ml of modified Chuken medium (pH 7.2). After shaking at 30 °C for 5 days, the culture broth was centrifuged (3000$\times$g, 5 min) and the supernatant was used for assay of GS activity.

Assay for GS inhibitory activity. Fourteen-day-old light-grown shoots (10 g) were washed and cut into 5 mm length. The sample was ground in a mortar with 15 ml of extract solution. The extract solution was composed of imidazole-HCl buffer (pH7.2; 50 mM), EDTA (pH7.2; 0.5 mM) and 2-mercaptoethanol (5 mM). After the extract was filtered through four layers of cheesecloth, the filtrate was centrifuged at 15,000 g for 15 min and the supernatant was used for the assay of GS activity.

GS activity was measured by the formation of γ-glutamylhydroxamate in the sythetase reaction.[46-54] The reaction mixtures contained enzyme (100 μl), sodium L-glutamate (50 μM), NH_2OH (pH7.2; 100 μM), NaATP (10 μM), $MgCl_2$ (20 μM), imidazole-HCl buffer (pH7.2; 50 μM), 2-mercaptoethanol (25 μM), and inhibitor, in a final volume of 1.0 ml. After incubation at 37 °C for 15 min, the reaction mixture was treated with 1.5 ml of ferric chloride reagent contained ferric chloride (0.37 M),

HCl (0.67 M) and TCA (0.20 M). The formation of γ-glutamylhydroxamate was measured at 530 nm (Shimazu UV 2200 spectrophotometer) and compared with a control in which no inhibitor was added. The standard curve was plotted in the manner described above by utilizing 1 ml samples of γ-glutamylhydroxamate (0.1 to 0.7 μmole).

Procedures for characterization of strain S-35

Morphological characterization. Morphological characteristics[90-100] were observed on several media described by Shirling and Gottlieb.[91] Incubation was carried out at 30 °C for 21 days.

Temperature of growth. Slants of Bennett's agar or yeast dextrose agar were inoculated, quickly heated or cooled to the desired temperature in water bath and then placed in water bath at the same temperature inside a constant-temperature incubator. The water level and Temperature of the water bath were carefully maintained. The cultures were examined for growth after 5 to 7 days at temperatures of 35 °C or above, and after 3 weeks at 10 °C.[101,102]

Hydrolysis of gelatin. Duplicate plates of the medium were streaked once across with each culture. After 5 days' incubation at 28 °C, one plate was covered with 8 to 10 ml of the following solution : HgCl$_2$, 15 g; concentrated HCl, 20 ml; distilled water, 100 ml. Hydrolysis of the gelatin[103] was measured by a clear zone underneath and around the growth. The second plate was incubated for 10 days before testing.

Hydrolysis of starch. The medium for determining hydrolysis of starch[103] was used with the substitution of 10 g of potato starch for gelatin. Duplicate plates were streaked and stored at 28 °C. After 5 days' incubation, one plate was flooded with 8 to 10 ml of 95 % ethanol or I_2 / KI solution; the second plate, after 10 days. A clear zone surrounding the growth indicated the extent of hydrolysis.

Milk peptonization and coagulation. Skim milk (instant nonfat milk) 10 g and brom cresol purple (BCP) were dissolved in 100 ml distilled water. The solution was then autoclaved, inoculated and incubated at 30 °C for 5 to 10 days.[97]

Melanoid pigment production. Production of melanoid pigments was determined on agar slants of peptone iron agar and tyrosine agar. Cultures used as inoculum source should be less than 3 weeks old. Duplicate slants were streaked and incubated at 30 °C for 4 to 7 days. The inoculated test tubes were compared with uninoculated controls. Cultures forming a brown to black diffusible pigment or distinct brown pigment modified by other color shall be recorded as positive. Absence of brown to black colors, or total absence of diffusible pigment, shall be recorded as negative for melanoid pigment production.[91]

Oxygen requirement. Oxgen requirement test[104] was determined on yeast-malt extract agar. Duplicate slants were streaked and incubated at 30 °C for 4 to 7 days.

Hydrogen sulfide production. Peptone-iron agar was employed in tube slants for determining the hydrogen sulfide reactions of the organisms.[105] Inoculum from actively growing cultures was used to streak the surface of the agar slants. After a short incubation period (15 to 20 hr) at 28 °C, the slants were observed. A pronounced bluish black discoloration of the medium surrounding the colonies indicated the production of hydrogen sulfide; whereas, those organism not producing hydrogen sulfide effected no change in the medium, or emitted only faint tints of other colors.

Reduction of nitrate. Cultures in nitrate broth were tested for the presence of nitrate after 5, 10, 14 days of incubation at 28 °C. One ml of broth culture was withdrawn aseptically and mixed with 3 drops of each of the following two solutions: (1) sulphanilic acid, 8 g; 5 N acetic acid, 1000 ml. (2) dimethyl-a-naphthylamine, 6 ml; 5 N acetic acid, 1000 ml. The appearence of a red color indicated the presence of nitrate.[103]

Salt tolerance. Salt tolerance was determined on yeast-malt extract agar containing NaCl prepared at concentration of 0, 3, 7, 10 and 14 %, respectively.[101] Duplicate slants were streaked and incubated at 30 °C for 4 to 7 days.

Sugar utilization. Chemically pure carbon sources were used for sugar utilization test. Carbon sources for this test are : D-glucose, L-arabinose, sucrose, D-xylose, l-inositol, D-mannitol, D-fructose, rhamnose, raffinose and cellulose.[90,91,94,95,106,107] These carbon sources should be sterilized without heat by the following method. An

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appropriate amount of the dry carbon source was weighed and spread as a shallow layer in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Ethylether was added to cover the carbohydrate. Ether was allowed to evaporate at room temperature under a ventilated fume hood over night. After all ether has evaporated, sterile distilled water was added asepticallt to make a 10 % w/v solution of the carbon source. After autoclaving the Pridham and Gottlieb[90] basal agar medium, cooled it to 60 °C and added sterile carbon source aspectically to give a concentration of approximately 1 %. The medium was agitated and poured 15 ml per dish into Petri dishes. Duplicate plates were streaked and incubated at 30 °C. After 20 days incubation, plates were observed and compared growth on a given carbon source with the two controls ; growth on basal medium alone, and growth on basal medium plus glucose.

Cell wall analysis. Strain S-35 was suspended in 5 ml of modified Chuken medium[108] and incubated with constant shaking at 30 °C for 1 day. The culture suspension was added to a 500-ml flask containing 200 ml modified Chuken medium and incubated with constant shaking at 30 °C for 5 days. The cells were killed with formalin (final concentration, 1 %) for 24 hr at room temperature and collected by centrifugation (6000$\times$g, 15 min). The cells were washed once in distilled water and once in 95 % ethanol and then dried by overnight drying at 45 °C. The dried cells were analyzed for diaminopimelic acid (DAP)[109-112] and carbohydrates[109] as described below.

The procedure was followed for the hydrolysis of whole cells preceding DAP analysis. Approximately 3 mg (dry weight) of cells was

placed into a small flask with 1 ml of 6 N HCl. The sealed flask was kept at 100 °C in oil bath for 18 hr. After cooling, the hydrolysate was filtered through Whatman no. 1 paper. The filtrate was evaporated to dryness and redissolved in 1 ml of distiled water, and taken to dryness again. This residue was dissolved in 0.3 ml of distilled water, and 2 µl was applied at the base line of the TLC sheet (Advantec No.50 filter paper). Ascending TLC was performed with the solvent system methanol-distilled water-6 N HCl-pyridine (80:26:4:10, v/v/v/v) for approximately 3.5 hr. After the chromatogram was air dried, spot were visualized by spraying with 0.2 % ninhydrin in acetone and heating at 110 °C for 5 min. As a DAP standard, 1 µl of 0.01 M DL-DAP (Sigma Chemical Co.), which contains both *meso*- and L-DAP isomers, was used. The DAP spots were seen as gray-green fading to yellow, with the L isomer moving ahead of the *meso* isomer.

The carbohydrate analysis was carried out the following method. Approximately 25 mg (dry weight) of cells was placed into an small flask with 1.5 ml of 1 N H_2SO_4. The sealed flask was heated at 100 °C in oil bath for 2hr. After cooling, the hydrolysate was transferred to a 15-ml centrifuge tube, and saturated barium hydroxide was added dropwise until the pH was between 5.2 and 5.5 (determined with pH paper). The precipitate was removed by centrifugation (3000 ҳ g, 10 min) and discarded. The supernatant fluid was evaporated under reduced pressure, and the residue was redissolved in 0.3 ml of distilled water; 1 µl of this hydrolysate was applied to the base line of the TLC sheet as well as two standard solution. The first contained galactose, arabinose, and xylose, each at 1 % concentration. The second solution contained rhamnose, mannose, glucose, and ribose, also each at 1 %. Ascending TLC was

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performed with the solvent system n-butanol-distilled water-pyridine-toluene (10:6:6:1, v/v/v/v) for approximately 4 hr. Spots were visualized by spraying the chromatogram with acid aniline phthalate (3.25 g of phthalic acid dissolved in 100 ml of water-saturated butanol plus 2 ml of aniline) and heating at 100 °C for 10 min. Hexose spots yellow after heating, and pentose spots were maroon. The carbohydrates migrated in the follwing sequence from origin : galactose, glucose, arabinose, mannose, xylose, ribose, and rhamnose.

Fermentation, isolation and structure analysis of S35-C2

Fermentation of strain S-35. One loopful of strain S-35 was aseptically transferred from an agar slant into 30-ml tube containing 5 ml of modified Chuken medium and cultured with shaking for 24 h at 30 °C. The culture was transferred into 500-ml Sakaguchi flask containing 200 ml of the same medium and cultured with shaking for 24 h at 30 °C. This culture was transferred into 500-ml Sakaguchi flask containing 600 ml of the same medium and cultured with shaking for 120 h at 30 °C.

Isolation and purification of S35-C2. The filtrate separated by centrifugation from a culture broth (30 liters) was adjusted to pH 9 with 5 N NH4OH, and activated charcoal (1 %, w/v) was added. The activated charcoal fraction was collected ,and dried at room temperature. This fraction was eluted with 80 % acidic methanol (pH 2). The eluate was concentrated under reduced pressure and dissolved with water. The solution was partitioned with chroloform and the organic

layer was concentrated under reduced pressure. The extract was put on a silica gel column eluted with chloroform-methanol. The fraction containing S35-C2 (chloroform-methanol, 99 : 1 v/v) was purified by crystallizing from EtOAc and obtained as colorless plates in yield of 7.2 mg / liter (Fig. 2).

Instrumental analyses. Instrumental analyses were performed using the following instruments: mp, Yanaco MP-S3 micro melting point apparatus; optical rotation, Horiba SEPA-200 high sensitive polarimeter; IR, Jasco FT/IR 7000; UV, Shimazu UV2200 UV-VIS recording spectrophotometer; 1H and 13C NMR spectra, JEOL JNM-GX270 FT-NMR spectrometer.
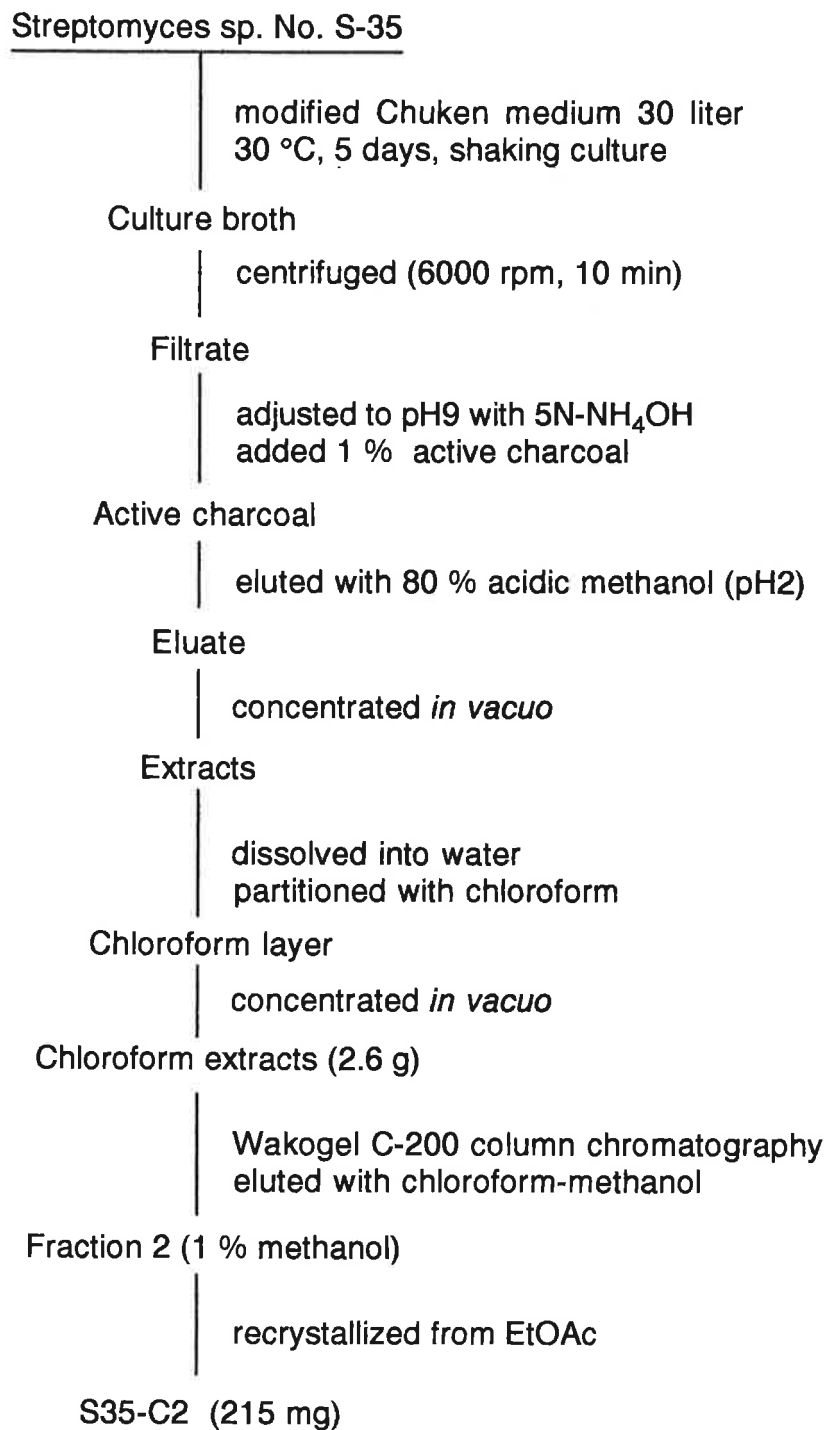
Streptomyces sp. No. S-35

```
                        │  modified Chuken medium 30 liter
                        │  30 °C, 5 days, shaking culture
                        │
    Culture broth
                        │  centrifuged (6000 rpm, 10 min)
                        │
    Filtrate
                        │  adjusted to pH9 with 5N-NH₄OH
                        │  added 1 % active charcoal
                        │
    Active charcoal
                        │  eluted with 80 % acidic methanol (pH2)
                        │
    Eluate
                        │  concentrated in vacuo
                        │
    Extracts
                        │  dissolved into water
                        │  partitioned with chloroform
                        │
    Chloroform layer
                        │  concentrated in vacuo
                        │
    Chloroform extracts (2.6 g)
                        │  Wakogel C-200 column chromatography
                        │  eluted with chloroform-methanol
                        │
    Fraction 2 (1 % methanol)
                        │  recrystallized from EtOAc
                        │
    S35-C2 (215 mg)
```

adjusted to pH9 with $5N\text{-}NH_4OH$

Fig. 2 Isolation Procedure of S35-C2

List of the culture media for strain S35

Culture medium for the production of S35-C2
Modified Chuken medium

Meat extract	1	g
Yeast extract	2.5	g
Peptone	10	g
D-glucose	20	g
$(NH_4)_2SO_4$	5	g
KCl	4	g
K_2HPO_4	0.2	g
$CaCO_3$	4	g
Distilled water	1000	ml
pH	7.2	

Culture media for identification of strain S-35

Trace salts solution

$FeSO_4. 7H_2O$	0.1	g
$MnCl_2. 4H_2O$	0.1	g
$ZnSO_4. 7H_2O$	0.1	g
Distilled water	100	ml

Sucrose-nitrate agar

Sucrose	30	g
$NaNO_3$	2	g
K_2HPO_4	1	g
$MgSO_4. 7H_2O$	0.5	g
KCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
Agar	15	g
Distilled water	1000	ml
pH	7.2	

Glucose-asparagine agar

D-glucose	10	g
L-asparagine	0.5	g
K_2HPO_4	0.5	g
Agar	15	g
Distilled water	1000	ml
pH	7.0	

Glycerol-asparagine agar

Glycerol	10	g
L-asparagine	1	g
K_2HPO_4	1	g
Trace salt solution	1	ml
Agar	15	g
Distilled water	1000	ml
pH	7.2	

Starch-yeast extract agar

Yeast extract	2	g
Soluble starch	10	g
Agar	15	g
Distilled water	1000	ml
pH	7.2	

Tyrosine agar

Glycerol	15	g
L-tyrosine	0.5	g
L-asparagine	1	g
K_2HPO_4	0.5	g
$MgSO_4. 7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4. 7H_2O$	0.01	g
Trase salts solution	1	ml
Agar	15	g
Distilled water	1000	ml
pH	7.2	

Nutrient agar

Peptone	5	g
Meat extract	5	g
NaCl	5	g
Agar	15	g
Distilled water	1000	ml
pH	7.3	

Yeast extract-malt extract agar

Yeast extract	4	g
Malt extract	10	g
D-glucose	4	g
Agar	15	g
Distilled water	1000	ml
pH	7.3	

Oatmeal agar

Oatmeal	20	g
Trace salts solution	1	ml
Agar	18	g
Distilled water	1000	ml
pH	7.2	

Medium for gelatin liquefaction

Peptone	5	g
Meat extract	3	g
Gelatin	4	g
Agar	15	g
Distilled water	1000	ml
pH	7.0	

Medium for starch hydrolysis

Peptone	5	g
Meat extract	3	g
Potato starch	20	g
Agar	15	g
Distilled water	1000	ml
pH	7.0	

Medium for milk peptonization and coagulation

Skim milk	10	g
Bromcresol purple	0.1	g
Distilled water	100	ml
pH	7.2	

Peptone-yeast extract iron agar for production of melanoid pigments

Peptone	15	g
Iron ammonium citrate	0.5	g
K_2HPO_4	1	g
$Na_2S_2O_3$	0.08	g
Yeast extract	1	g
Agar	15	g
Distilled water	1000	ml
pH	7.2	

Medium for hydrogen sulfide production

Meat extract	3	g
Yeast extract	3	g
Peptone	20	g
D-glucose	1	g
Lactose	10	g
Sucrose	10	g
$FeSO_4. 7H_2O$	0.2	g
NaCl	5	g
$Na_2S_2O_3. 5H_2O$	0.3	g
0.2 % phenolred solution	12	ml
Agar	20	g

Distilled water	1000	ml
pH	7.4	

Medium for reduction of nitrate

Peptone	5	g
Meat extract	3	g
KNO_3	1	g
Distilled water	1000	ml
pH	7.0	

Medium for sugar utilization

Pridham and Gottlieb trace salt

$CuSO_4.\ 5H_2O$	0.64	g
$FeSO_4.\ 7H_2O$	0.11	g
$MnCl_2.\ 4H_2O$	0.79	g
$ZnSO_4.7H_2O$	0.15	g
Distilled water	100	ml

Basal mineral salts agar

$(NH_4)_2SO_4$	2.64	g
KH_2PO_4	2.38	g
$K_2HPO_4.\ 3H_2O$	5.65	g
$MgSO_4\cdot\ 7H_2O$	1.00	g
Pridham & Gottlieb trace salt	1	ml
Agar	15	g
Distilled water	1000	ml

pH   7.0

Results and Discussion

Effects of twelve chemicals on sterility in rice (in 1990)[113]

The twelve chemicals to be tested were applied at a concentration of 1,000 mg/l at meiosis in 1990 (Table1). Kasugamycin and AOA induced high levels of sterility in rice of 89.4 % and 95.3 %, respectively. However, the culm length of rice plants treated with AOA was shorter than that of the control. GA_3 for maize[114-116] and benzotriazole for wheat[117] as male sterilants produced no marked effect on sterility for rice. ACC[118-122] as an immediate precursor of ethylene[29] and NAA induced as the same levels of sterility as that in the control. Spermine and MGBG,[123,124] which are related to the regulation of ethylene production, induced sterility similar to that in the control. Azetidine-2-carboxylic acid ,[125] which is an analogue of azetidine-3-carboxylic acid that induced male sterility in wheat,[126] produced no effect on sterility in rice. D-Cycloserine, hydroxyproline,[127] and α,α'-dipyridil,[128] which are inhibitors of proline metabolism, were applied at meiosis, but these did not induce effective sterility in rice. It is well known from biochemical studies that the proline content of such cytoplasmic male sterile anthers as rice,[129,130] wheat, maize,[131] sorghum,[132] and suger beet[133] is less than that of normal anthers. The inhibitors of proline metabolism were applied thus at meiosis, but they did not effectively induce sterility in rice.[129,130]

Table 1. Mean Number of Seeds, Sterility, Spike Length, Culm Length and Weight of Ripe Seeds of "Yamabiko" Rice as Affected by Twelve Chemicals at Meiosis

Chemical[a]	Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of raped seeds (g X $1,000^{-1}$)
Kasugamycin	73	89.4 d[b]	17.2	48.2	25.4
AOA	57	95.3 d	15.9	28.8	--
GA$_3$	52	26.3 b	21.7	79.5	25.4
NAA	55	14.2 a	16.3	52.9	26.0
Benzotriazole	66	26.3 b	16.7	46.9	24.9
ACC	62	9.1 a	17.0	54.1	26.0
Spermine	69	9.1 a	17.5	53.1	24.3
MGBG	69	10.0 a	17.9	54.0	--
ACA	70	8.9 a	17.2	56.6	26.2
D-Cycloserine	71	12.1 a	17.8	54.3	26.9
Hydroxyproline	70	9.9 a	17.5	53.7	25.5
α,α'-Dipyridil	71	9.4 a	18.1	53.5	26.5
Control	71	10.6 a	17.0	51.4	25.5

[a] Applied at a concentration of 1,000 mg/l.

[b] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

Effects of kasugamycin and AOA on sterility in rice (in 1991)

Kasugamycin and AOA (Fig. 3)were found to induce effective sterility in rice in 1990, so these two chemicals were applied to rice plants in 1991 (Fig. 4, 5) to find the optimum application period and dosage for the induction of sterility.

Kasugamycin

$$NH_2-O-CH_2-COOH$$
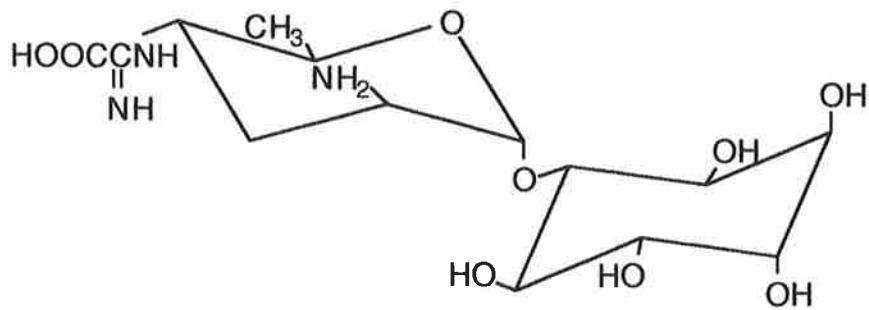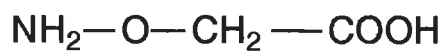
AOA

Fig. 3 The Structures of Kasugamycin and AOA
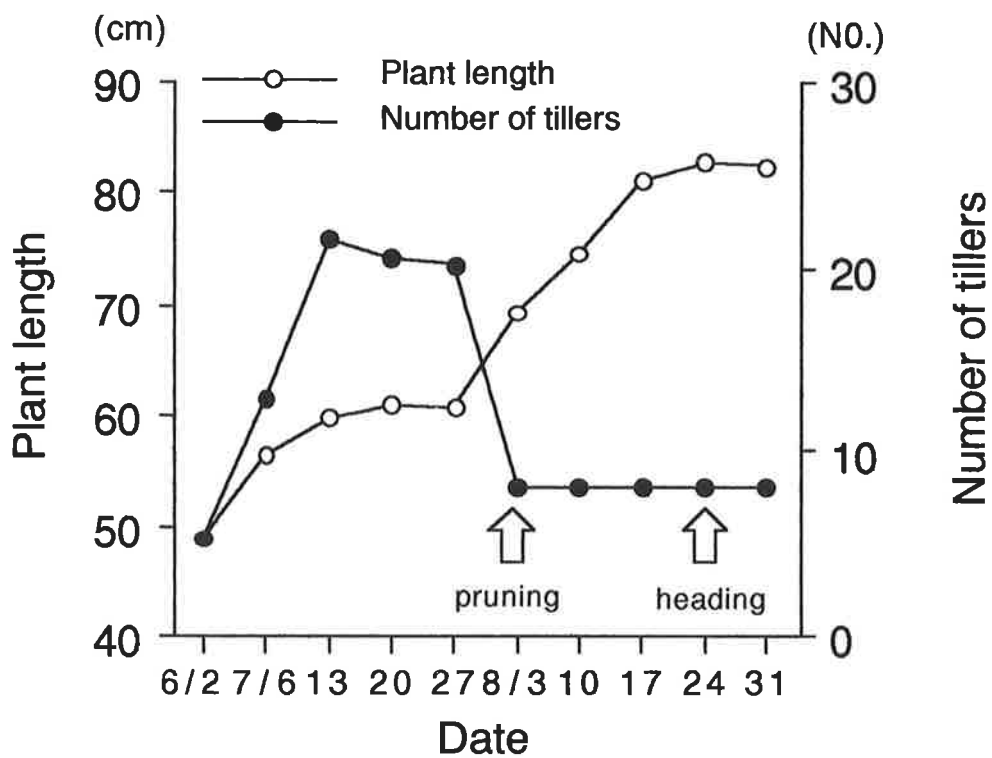
Fig. 4 Growth Process of *Oryza sativa* (cv Yamabiko) in 1991
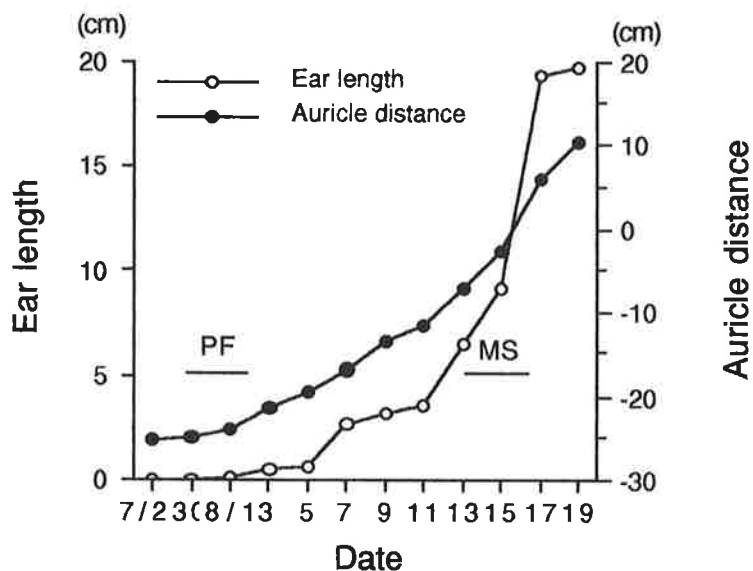
Fig. 5 Growth Process of Ear Length and Auricle Distance

PF, Panicle Formation stage; MS, Meiosis

In 1991 (Table 2), kasugamycin also induced a high level of sterility in rice at a concentration of 1,000 mg/l at meiosis, but its ratio for sterility was similar to that of the control when applied at the same concentration at the panicle formation stage. When AOA was applied at the concentration of 100 and 1,000 mg/l at the panicle formation stage, the ratio of sterility was 81.8 % and 100 %, respectively. However, the number of seeds, spike length, culm length and weight of ripe seeds in the rice plants treated with AOA at both concentrations were inferior to those of the control. When AOA was applied at the same concentrations as those mentioned above at meiosis, the ratio of sterility was 35.3 % and 97.2 %, respectively. The spike length and culm length of rice plants treated with AOA were shorter than those of the control when applied at a concentration of 1,000 mg/l. These results show that kasugamycin was effective for inducing high sterility in rice at meiosis without crop injury, and that

3 1

AOA was effective for inducing complete sterility in rice at the panicle formation stage.

Table 2. Mean Number of Seeds, Sterility, Spike Length, Culm Length and Weight of Ripe Seeds of "Yamabiko" Rice as Affected by Kasugamycin and AOA at Two Growth Stages and at Two Rates

Chemical			Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of ripe seeds (g X $1,000^{-1}$)
Panicle formation stage							
Kasugamycin	100	mg/l	77	14.3 a[a]	18.8	51.7	25.1
	1000	mg/l	81	23.1 a	18.1	49.3	23.4
AOA	100	mg/l	46	81.8 c	14.5	38.5	20.4
	1000	mg/l	21	100 c	10.4	24.1	-
Meiosis							
Kasugamycin	100	mg/l	67	13.0 a	18.3	50.5	24.6
	1000	mg/l	68	51.3 b	18.3	46.3	22.2
AOA	100	mg/l	63	35.3 b	17.3	42.4	22.7
	1000	mg/l	63	97.2 c	14.8	24.2	24.2
Control			64	13.6 a	17.4	49.0	22.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

In this investigation, kasugamycin and AOA were found to induce high levels of sterility in rice. Kasugamycin induced high sterility in rice at a concentration of 1,000 mg/l at meiosis, while AOA induced

complete sterility in rice at a concentration of 1,000 mg/l at the panicle formation stage. These results show that AOA was the most effective for inducing sterility in rice among the chemicals tested. However, rice plants treated with AOA suffered from crop injury such as straighthead. This phenomenon suggests that AOA inhibited plant senesence, since AOA is an inhibitor of ACC synthase[30] which produces ACC which is an immediate precursor of ethylene (Fig. 6).[29]
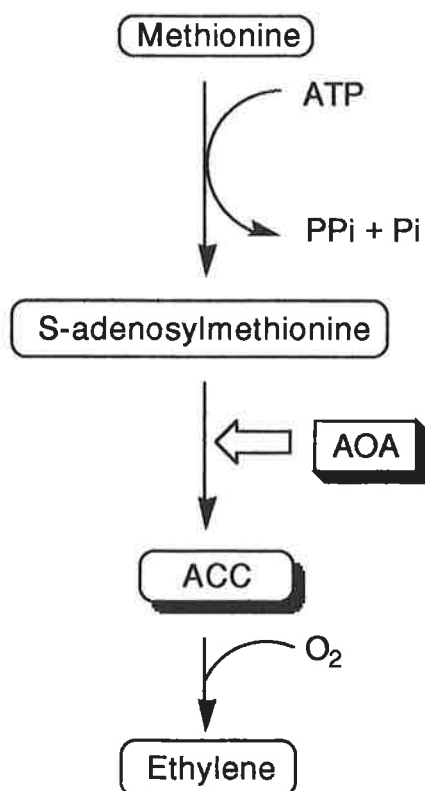
Fig. 6 Regulation of Ethylene Biosynthesis

Kasugamycin had a weaker activity for inducing sterility in rice than AOA. Rice plants treated with kasugamycin were similar to the control in the number of seeds, spike length, culm length and weight of ripe seeds, while rice plants treated with AOA were inferior to the control in these respects. These results suggest the possibility of inducing a high level of sterility in rice without crop injury if more effective kasugamycin analogues can be developed.

Sex expression of " Yamabiko " rice plants treated with AOA

Table 3 shows sex reversal from stamens to pistils in a glumous flower of " Yamabiko " rice plants when AOA was applied at a concentration of 1.000 mg/l at the panicle formation stage. Although the total number of stamens and pistils per a glumous flower which were affected by AOA application was the same as those of the control, the number of pistils affected by AOA was 3.4 times as many as that of the control. These results show that a stamen was changed to a pistil in a glumous flower when AOA was applied at a concentration of 1,000 mg/l at the panicle formation stage. AOA induced the conversion of sex expression from a stamen to a pistil in a glumous flower of the rice plant. Although ACC is known to convert sex expression from staminate flowers to pistillate flowers in cucumber,[134,135] the conversion of sex expression in rice plants by AOA is proved by this investigation. It is suggested that endogeneous ACC or ethylene may play an important role in sex expression in rice at the initial reproductive stage (Fig. 6).

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Table 3. Sex Expression of "Yamabiko" Rice Plants
as Affected by AOA Application

	Number of stamens[a]	Number of pistils[a]
AOA (1,000 mg/l)	3.6 ± 0.5	3.4 ± 0.5
Control	6 ± 0	1 ± 0

[a] Data presented are the mean of three replications
of seven glumous flowers

Interactive effect between kasugamycin and plant hormones on sterility

As kasugamycin induced high sterility in rice without crop injury, the interactive effect between kasugamycin and plant hormones on sterility was tested (Table 4). Treatment by kasugamycin with ethrel induced higher sterility than that with kasugamycin treatment alone. Treatment by kasugamycin with NAA or BA, in contrast, decreased the ratio of sterility when compared with that by kasugamycin treatment alone. In particular, treatment by kasugamycin with NAA caused the same level of sterility as that in the control. In the interaction between kasugamycin and the four plant hormones of GA_3, NAA, BA, and ethrel, fertility was restored to rice when NAA was applied simultaneously with kasugamycin. However, it is not clearly understood yet what is the mode of action of NAA on the restoration of fertility.

Table 4. Mean Number of Seeds, Sterility, Spike Length, Culm Length and Weight of Ripe Seeds of "Yamabiko" Rice as Affected by Kasugamycin and One of Four Plant Hormones at Meiosis

Chemical		Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of ripe seeds ($g \times 1,000^{-1}$)
Kasugamycin	1000 mg/l	68	51.3 b[a]	18.3	46.3	22.2
+ NAA	300 mg/l	54	22.7 a	17.7	50.1	22.7
+ BA	300 mg/l	50	30.2 a	17.3	45.6	22.8
+ GA3	300 mg/l	55	62.2 b	19.5	58.1	25.3
+ ethrel	300 mg/l	48	87.2 c	15.8	41.4	24.4
Control		64	13.6 a	17.4	49.0	22.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

Effects of inhibitors of protein synthesis on sterility

Since kasugamycin is well known as an inhibitor of protein synthesis,[28] erythromycin,[136,137] kanamycin,[138-141] tetracycline,[142] and spectinomycin[143-147] which are other inhibitors of protein synthesis, were tested for their effect on sterility in rice (Table 5). However, none of these four chemicals induced high sterility in rice when applied at a concentration of 1,000 mg/l at meiosis.

Table 5. Mean Number of Seeds, Sterility, Spike Length, Culm Length and Weight of Ripe Seeds of "Yamabiko" Rice as Affected by Protein Inhibitors at Meiosis

Chemical[a]	Number of seeds (no./spike)	Sterility (%)	Spike lenght (cm)	Culm length (cm)	Weight of ripe seeds ($g \times 1,000^{-1}$)
Kasugamycin	68	51.3 b[b]	18.3	46.3	24.6
Erythromycin	66	12.5 a	18.5	50.7	23.8
Kanamycin	60	16.8 a	17.9	51.9	22.2
Tetracycline	54	27.5 a	17.7	48.7	23.7
Spectinomycin	68	16.0 a	18.3	49.5	23.4

[a] Applied at a concentration of 1,000 mg/l.

[b] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

Kasugamycin as an anti-rice blast antibiotic was isolated from *Streptomyces kasugaensis* in 1965.[27] Subsequently, it has been shown that kasugamycin inhibited protein synthesis of *Pyricularia oryzae* and *E.coli* (Fig. 7).[28] Based on this action, erythromycin, kanamycin, tetracycline, and spectinomycin, which are inhibitors of protein synthesis, were applied at meiosis, but these had little effect to induce sterility in rice. These results suggest that kasugamycin as an inhibitor of protein synthesis is a specific inducer of sterility in rice.
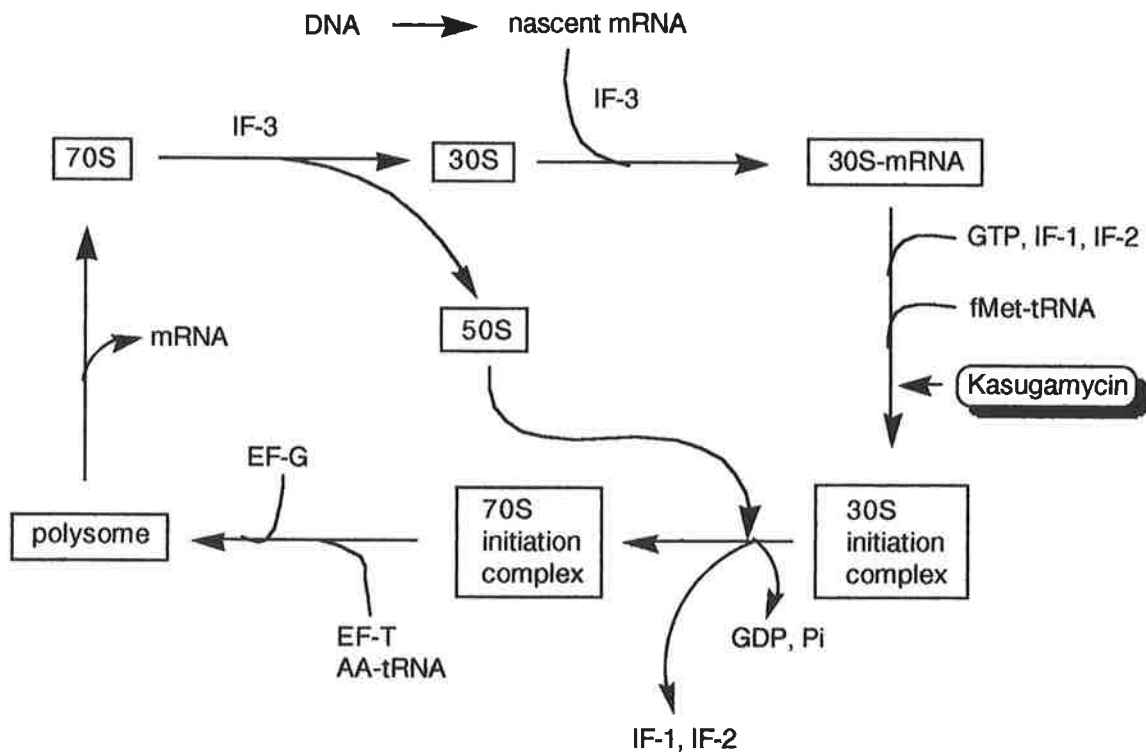
Fig. 7 Active Site of Kasugamycin in Protein Synthesis

30S, 50S, 70S : ribosome; IF-1, -2, -3 : initiation factors
EF-G, -T : elongation factors

Effects of inhibitors of nucleic acid synthesis on sterility in rice

Rifampicin,[148-150) guanidine thiocyanate,[151-153) and 1-(2-tetrahydrofuryl)-5-fluorouracil which are the inhibitors of nucleic acid synthesis, were tested for their effect on sterility in rice (Table 6). However, none of these three chemicals induced high sterility in rice when applied at a concentration of 1,000 mg/l at meiosis. By the way, it

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is known that self-incompatibility in *Nicotiana alata* is related to ribonuclease activity of style S-glycoproteins.[154-162] Based on this action, rifampicin as an RNA polymerase inhibitor, guanidine thiocyanate as an RNase inhibitor, and 1-(2-tetrahydrofuryl)-5-fluorouracil as an DNA polymerase inhibitor were applied at meiosis, but these had little effect to induce sterility in rice. These results suggest that the applications of inhibitors of nucleic acid have little effect against sterility in rice.

Table 6. Mean Number of Seeds, Sterility, Spike Length and Culm Length of "Yamabiko" Rice as Affected by Inhibitors of nucleic acid synthesis at Meiosis

Chemical	Number of seeds (no./spike)	Sterility (%)		Spike length (cm)	Culm length (cm)
Rifampicin 1000 mg/1	61	23.1	a[a]	18.3	51.0
Guanidine thiocyanate	52	23.1	a	17.0	47.8
1-(2-tetrahydrofuryl)-					
5-fluorouracil	52	18.7	a	17.4	48.2
Control	64	13.6	a	17.4	49.0

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

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Effect of bialaphos on sterility in rice (in 1991)[163]

When bialaphos was applied at meiosis (Table 7), 99.0 % sterility was induced by 0.1 mM bialaphos. Furthermore, pollen grains were little stained with acetocarmine solution (Fig. 8), when observed by microscopy. The result of acetocarmine staining suggested that sterility induced by bialaphos treatment was caused by inhibition of pollen growth. On the other hand, rice plants treated with bialaphos were inferior to the control plants with regard to number of seeds, spike length and culm length. As high sterility in rice is induced at a low concentration (0.1 mM) of bialaphos, it may be regarded as an effective sterilant if it does not injure the crop.

Table 7. Mean Number of Seeds, Sterility, Spike Length and Culm Length of "Yamabiko" Rice as Affected by Bialaphos at Meiosis

Chemical	Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)
Bialaphos 0.1 mM	28	99.0 b[a]	12.5	40.3
Control	64	13.6 a	17.4	49.0

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.
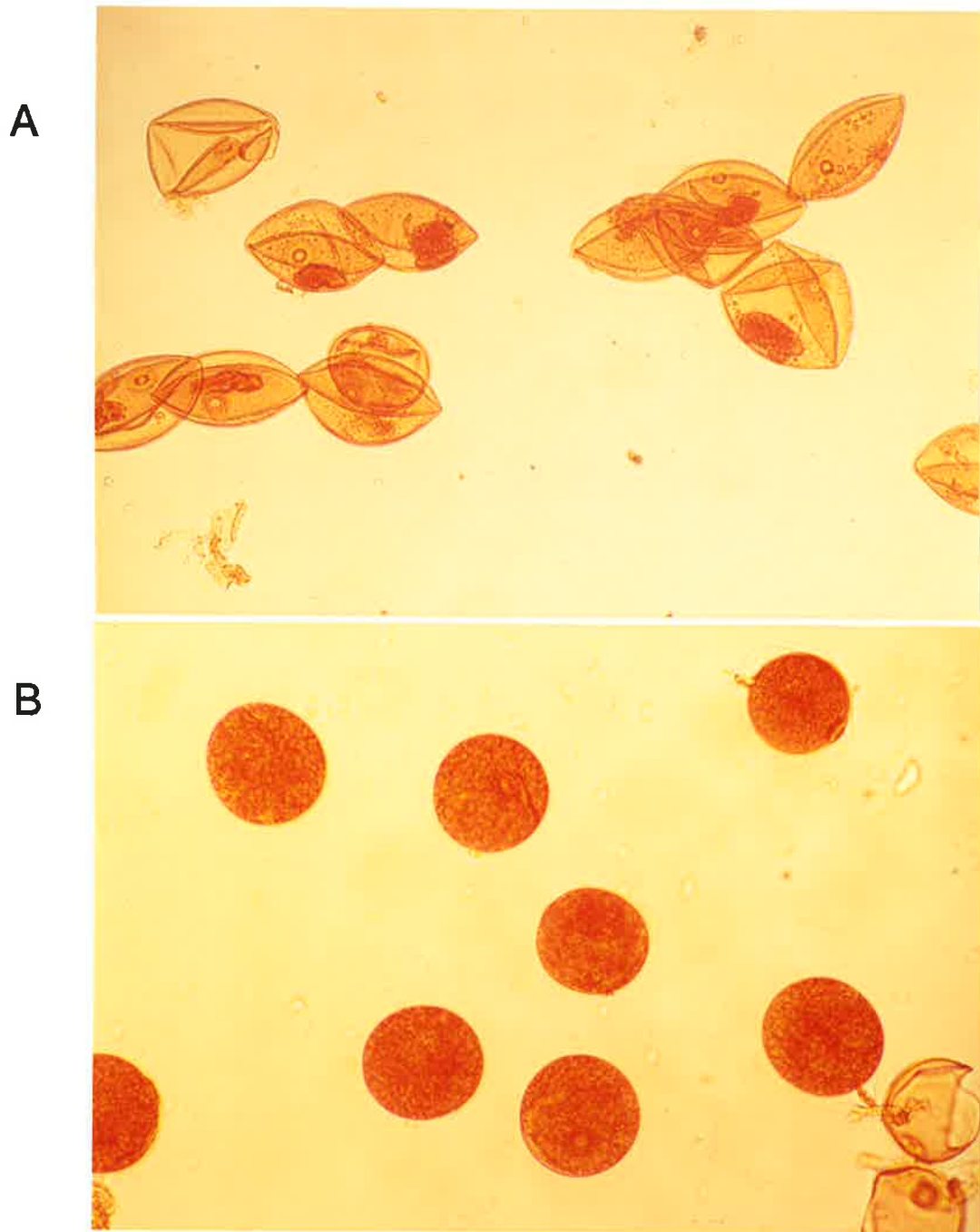
Fig. 8 Rice Pollen Grains Stained with Acetocarmine Solution (X200)

A: Bialaphos Treatment (0.1 mM) B: Control

Biochemical characteristics of rice sterility treated with bialaphos

Effect of bialaphos on GS activity, in vitro

The assay was carried out on GS extracted from shoots of the rice plants. GS activity was determined by the γ-glutamylhydroxamate procedure.[48] At the concentrations tested in this experiment (0-3 mM) (Fig. 9), bialaphos did not inhibit GS *in vitro*. However, L-PTC inhibited GS by 81 % at a concentration of 0.1 mM. Furthermore, L-PTC inhibited completely GS at a concentration of 1 mM. Since it is known in Japanese barnyard millet (*Echinochloa utilis* OHWI)[45] that bialaphos is metabolized into L-PTC, it is suggested that L-PTC is the real inhibitor of GS from rice plants and the real inducer of sterility in rice.
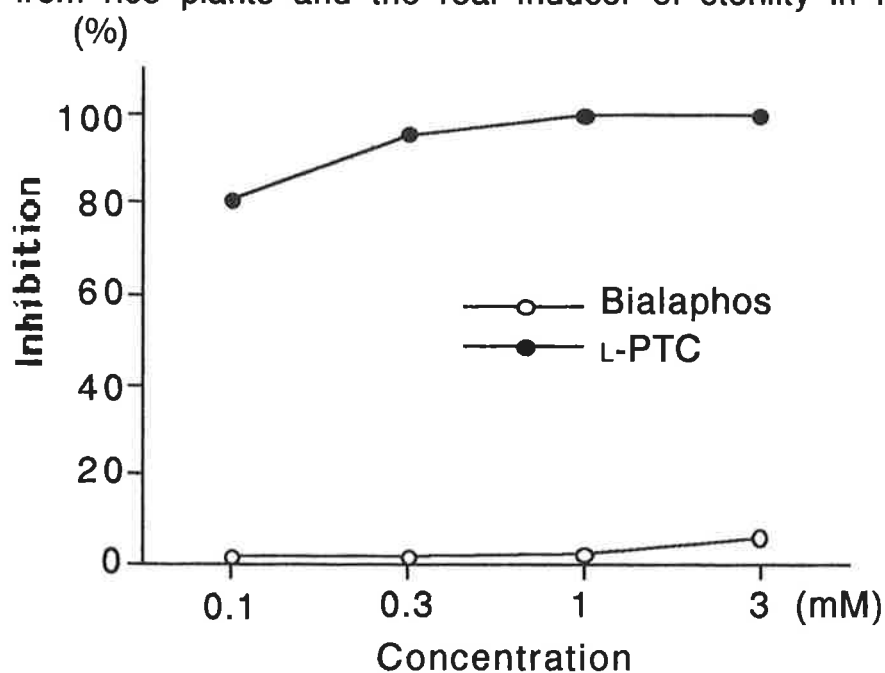
Fig. 9 Effects of Bialaphos and L-PTC on Glutamine Synthetase from Shoots of Rice Plants, *in vitro*

Changes of free amino acids in anthers

The total contents of free amino acids in untreated and bialaphos-treated anthers were 15.41 nM and 1.36 nM per anther, respectively. The rate of proline was about a half of free amino acids in control anthers. Proline is utilized in new protein synthesis[164] and can be used as an energy source;[165] however, the role of proline in connection with pollen fertility is not clearly understood.[131,166]

The untreated anthers had about twice as much proline as the anthers treated with bialaphos, while the treated anthers had about 4~6 times more aspartic acid and glutamic acid than the untreated anthers (Table 8). The decrease of proline in bialaphos treated anthers was similar to that found for cytoplasmic male sterile anthers as of rice,[129,130] wheat, maize,[131] sorghum[132] and sugar beet.[133] The characteristic changes in the free amino acids in anthers treated with bialaphos are the increases of glutamic acid and aspartic acid. Bialaphos, an inhibitor of GS, inhibits the conversion of glutamic acid to glutamine.[45] As a result of glutamine deficiency, the conversion of aspartic acid to asparagine is inhibited because glutamine plays an important role as the amide nitrogen donor in asparagine synthesis in rice plants.[167,168] This in turn means that proline synthesis is also inhibited, since asparagine is a precoursor of proline.[169,170] Therefore, the decrease of proline and the increase of glutamic acid and aspartic acid which occur in sterile anthers treated with bialaphos is easily explained (Fig. 10).

Table 8. Free Amino acids Composition (%) of Anther Treated with Bialaphos

Amino acids	Bialaphos	Control
Aspartic acid	10.14	1.75
Threonine	2.93	3.08
Serine	3.09	3.48
Glutamic acid	6.17	1.80
Proline	20.50	44.72
Glycine	5.95	0.84
Alanine	9.11	10.01
Cysteine	tr.	tr.
Valine	5.73	2.82
Methionine	1.83	4.58
Isoleucine	3.10	2.52
Leucine	3.09	5.62
Tyrosine	4.48	3.42
Phenylalanine	3.01	3.02
Histidine	11.17	6.23
Lysine	4.78	2.12
Arginine	4.92	3.99

tr., trace
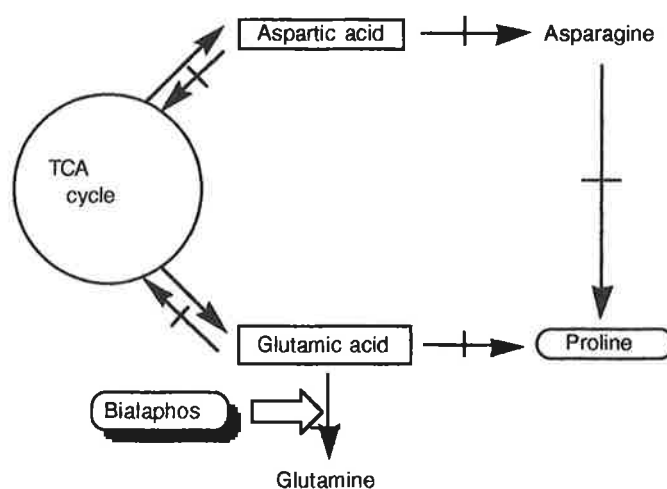
Fig. 10 The Possible Pathway of Abnormal Amino acid Metabolism in the Sterile Anthers Treated with Bialaphos

☐ : Accumulation, ⬭ : Decrease

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Rice sterility induced by inhibitors of glutamine synthetase

Effects of inhibitors of glutamine synthesis on sterility in rice (in 1992)

Bialaphos was found to induce effective sterility in rice in 1991. Three inhibitors of glutamine synthesis, bialaphos,[41-46] L-phosphinothricin (L-PTC)[40] and L-methionine sulfoximine (L-MSO) ,[69,70,171-173] were applied to rice plants in 1992 to find the optimum application dosage for the induction of sterility (Fig. 11).

Bialaphos

$$CH_3 - \underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{P}} - CH_2 - CH_2 - \underset{\underset{H}{|}}{\overset{\overset{NH_2}{|}}{C}} - CONH - \underset{\underset{H}{|}}{\overset{\overset{CH_3}{|}}{C}} - CONH - \underset{\underset{H}{|}}{\overset{\overset{CH_3}{|}}{C}} - COOH$$

L-PTC (L-phosphinothricin)

$$CH_3 - \underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{P}} - CH_2 - CH_2 - \underset{\underset{H}{|}}{\overset{\overset{NH_2}{|}}{C}} - COOH$$

L-MSO (L-methionine sulfoximine)

$$CH_3 - \underset{\underset{NH}{\overset{\|}{}}}{\overset{\overset{O}{\|}}{S}} - CH_2 - CH_2 - \underset{\underset{H}{|}}{\overset{\overset{NH_2}{|}}{C}} - COOH$$
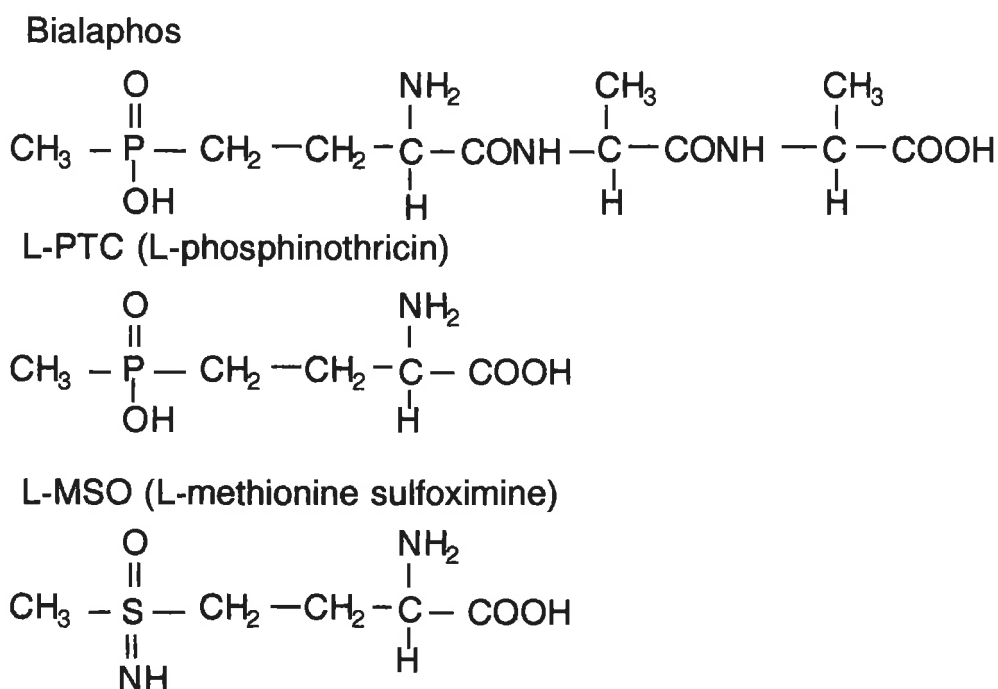
Fig. 11 The Structures of Bialaphos, L-PTC, and L-MSO

In 1992 (Table 9), bialaphos induced a high level of sterility in rice at a concentration of 0.1 mM at meiosis. L-PTC was also induced a high level of sterility in rice at a concentration of 0.1 mM at meiosis. The number of seeds, spike length and culm length in the rice plants treated with bialaphos and L-PTC at a concentration of 0.1 mM were slightly inferior to those of the control. Bialaphos induced complete sterility in rice at a concentration of 0.3 mM at meiosis, but rice plants withered before anthesis by treatment with L-PTC at a concentration of 0.3 mM. These results indicate that the effective dosage for the induction of sterility is in the narrow range of the concentrations from 0.1 mM to 0.3 mM.

Table 9. Mean Number of Seeds, Sterility and Spike Length of "Yamabiko" Rice as Affected by Three Chemicals at Meiosis

Chemical		Number of seeds (no./spike)	Sterility (%)		Spike length (cm)	Culm length (cm)	Weight of ripe seeds (g, 1000⁻¹)
Bialaphos	0.01 mM	63	5.3	a[a]	16.9	57.8	26.8
	0.03	66	3.6	a	17.3	57.0	25.7
	0.1	48	94.8	b	15.2	52.4	-
	0.3	18	100	b	10.8	46.0	-
L-PTC	0.01 mM	67	5.4	a	17.2	60.8	25.7
	0.03	59	6.0	a	16.5	58.2	26.0
	0.1	43	95.4	b	14.7	44.7	-
	0.3	-	-		-	-	-
L-MSO	0.01 mM	68	4.6	a	17.2	59.9	25.2
	0.03	71	5.4	a	17.7	60.1	25.5
	0.1	62	3.2	a	16.9	59.1	25.0
	0.3	58	2.8	a	17.1	58.5	24.9
Control		68	4.4	a	16.8	55.5	25.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

When the stamens and the pistils in the glumous flowers treated with 0.1 mM of bialaphos were observed by microscopy and compared with the untreated control before anthesis (Fig. 12), the shape of the pistil treated with bialaphos was the same as that of control. However, all stamens treated with bialaphos became albinos, and pollen grains in the anthers were little stained with acetocarmine solution and iodine-potassium iodide (IKI) solution, respectively (Fig. 8). The results of IKI staining suggested that pollen sterility induced by bialaphos was related to the absence or the decrease of pollen carbohydrate content. The results from the observation of the glumous flowers suggest that the growth of the pistils treated with bialaphos is normal and the stamens alone are inhibited the normal growth by treatment with bialaphos.

Fig. 12 Stamen and Pistil in the Glumous Flower Treated with Bialaphos

Figure 13 shows the panicles after maturation. The color of the panicles, which was sterilized by treatment with 0.1 mM of bialaphos and L-PTC, is pale green. The results suggest that the phenomenon occurs by the disaccumulation of nutrients in panicles.

Fig. 13 The Panicles Treated Bialaphos, L-PTC, and L-MSO
(After Maturation)

Effect of glutamine on sterility in rice treated with bialaphos

Bialaphos inhibits glutamine synthetase activity and is a potent herbicide for a wide range of weeds.[41-46] Glutamine has been reported to antagonize bialaphos through its action mechanism in inhibiting growth of pollen tubes of *Camellia japonica* L..[174] This suggest that action mechanism of bialaphos involves the GS inhibition and the shortage of glutamine. However, an exogenous application of glutamine does not antagonize bialaphos in Japanese barnyard millet (*Echinochloa utilis* OHWI).[45] In order to examine the interaction between bialaphos and glutamine on sterility in rice, glutamine applicated at the concentrations of 1 to 100 mM to rice plants simultaneously treated with bialaphos (Table 10).

Table 10. Mean Number of Seeds, Sterility and Spike Length of "Yamabiko" Rice as Affected by Bialaphos and Glutamine at Meiosis

Chemical		Number of seeds	Sterility	Spike length	Culm length	Weight of ripe seeds
		(no./spike)	(%)	(cm)	(cm)	$(g, 1000^{-1})$
Bialaphos	0.1 mM	48	94.8 b[a]	15.2	52.4	-
+ Glutamine	1	47	93.8 b	15.2	54.4	-
	10	42	95.6 b	15.0	51.7	-
	100	59	6.5 a	17.0	57.0	25.3
Control		68	4.4 a	16.8	55.5	25.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

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Treatment by bialaphos with glutamine at the concentrations of 1 and 10 mM induced the same level of sterility as that of bialaphos treatment alone. However, treatment by bialaphos with glutamine at a concentration of 100 mM caused the same sterility as that in the control. In the interaction between bialaphos and glutamine, fertility was restored to rice when glutamine was applied simultaneously with bialaphos at a concentration of 100 mM. These results suggest that the restoration of fertility caused by glutamine treatment resembles the phenomenon in the growth of pollen tubes of *Camellia japonica* L.[174] treated with bialaphos and glutamine, and that GS inhibition induced by bialaphos treatment is the reversible reaction by the interaction between bialaphos and glutamine.

Effects of ammonia and ammonium sulfate on sterility in rice

The endogenous ammonia accumulation as the result of GS inhibition in panicles of rice plants was considered a high sterility in rice. For this reason, effects of ammonia and ammonium sulfate on sterility were examined.

Ammonia and ammonium sulfate were applied at a concentration of 10 mM at meiosis, but these chemicals did not induce a high sterility in rice (Table 11). These results suggest that rice sterility is not caused by the exogenous application of ammonia[175,176] but caused by the endogenous ammonia.

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Table 11. Mean Number of Seeds, Sterility and Spike Length of "Yamabiko" Rice as Affected by Bialaphos, Ammonia and Ammonium sulfate at Meiosis

Chemical		Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of ripe seeds $(g\ 1,000^{-1})$
Bialaphos	0.1 mM	48	94.8 b[a]	15.2	52.4	-
Ammonia	10	62	6.1 a	16.7	55.2	25.1
Ammonium sulfate	10	64	5.5 a	17.3	57.2	24.6
Control		68	4.4 a	16.8	55.5	25.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

Effect of DCMU on sterility in rice treated with bialaphos

Since it was known that L-MSO[69,70] and tabtoxin[177-181] were GS inhibitors and that L-MSO- and tabtoxin-induced chlorosis and ammonium accumulation were light-dependent reactions which could be inhibited by dichlorodimethyl phenylurea (DCMU),[70,181] effect of DCMU on sterility in rice treated with bialaphos was examined.

Treatment by bialaphos with 1 mM of DCMU caused higher sterility than that with bialaphos treatment alone. Furthermore, the number of seeds, spike length and culm length in the rice plants treated with bialaphos and DCMU were inferior to those with bialaphos treatment alone (Table 12).

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Table 12. Mean Number of Seeds, Sterility and Spike Length of "Yamabiko"
Rice as Affected by Bialaphos, DCMU and Orthovanadate at Meiosis

Chemical		Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of ripe seeds (g 1000^{-1})
Bialaphos	0.1 mM	48	94.8 b[a]	15.2	52.4	-
+ DCMU	1	32	100 b	13.6	43.8	-
Orthovanadate	10	66	5.5 a	17.3	54.3	23.6
Control		68	4.4 a	16.8	55.5	25.3

[a] Means in a column followed by the same letter are not significantly
different at the 5 % level of significance by Duncan's Multiple Range
Test.

Physiolosical and biochemical characteristics of sterility in rice

Effects of GS inhibitors on GS activity, in vitro

The assay was carried out on GS extracted from panicles of the rice plants. GS activity was determined by the γ-glutamylhydroxamate procedure. At the concentrations tested in this experiment (0-0.3 mM) (Fig. 14), bialaphos did not inhibit GS *in vitro*. However, L-PTC inhibited GS by 96 % at a concentration of 0.1 mM. Furthermore, L-PTC inhibited completely GS at a concentration of 0.3 mM. L-MSO inhibited GS by 41 % and 61 % at the concentrations of 0.1 mM and 0.3 mM, respectively.
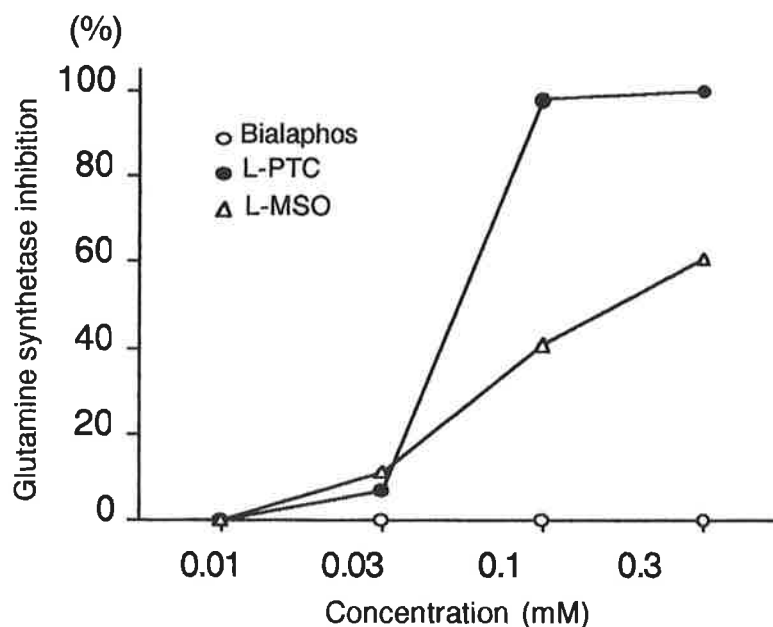
Fig.14 Effects of Bialaphos, L-PTC and L-MSO on Glutamine Synthetase of Rice Plants, *in vitro*

Glutamine synthetase activity and free ammonia content in panicles

The decrease of GS activity was observed in the panicles of rice after treatments with 0.1 mM of bialaphos and L-PTC (Fig. 16). The complete inhibition of GS activity was caused 24 hr after treatments, and this inhibition continued until anthesis. However, L-MSO did not inhibit GS activity until anthesis, and GS activity in panicles treated with L-MSO at a concentration of 0.1 mM was similar to that of control. In according to the inhibition of GS activity, the increase of free ammonia content was observed in the panicles of rice after treatments with 0.1 mM of bialaphos and L-PTC (Fig. 15, 16). The accumulation of ammonia was continued until anthesis.
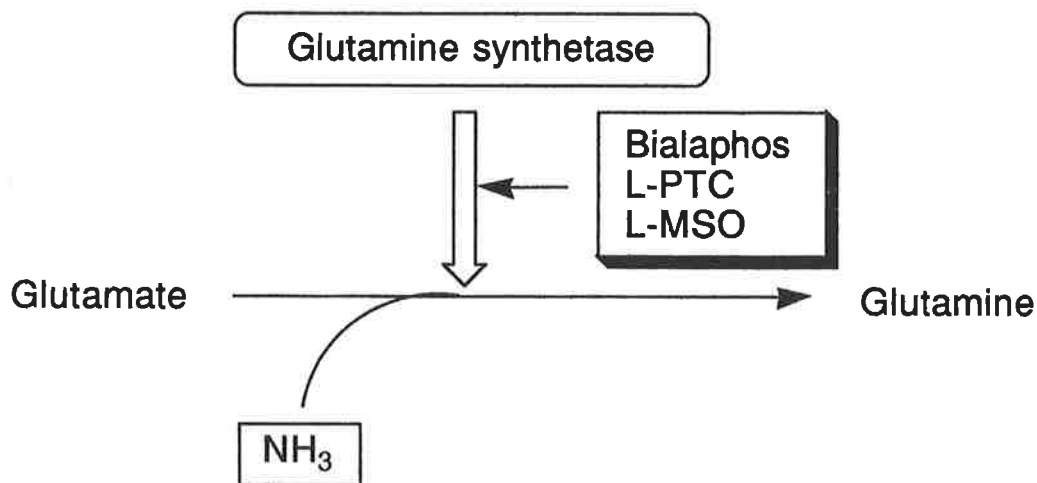
Fig. 15 Action Mechanism of GS Inhibitors
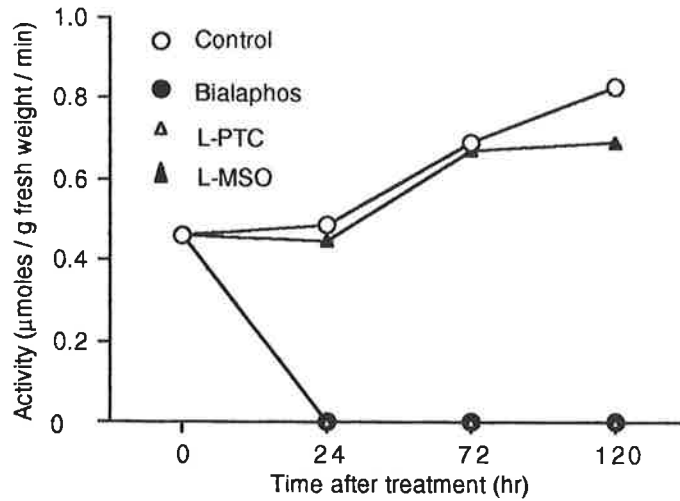
Time Course of Glutamine Synthetase Activity in Panicles
of Rice Plants Treated with Bialaphos, L-PTC and L-MSO
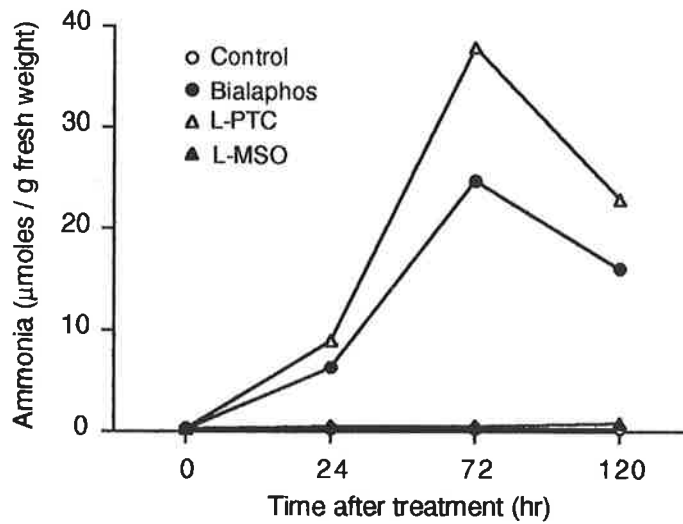
Time Course of Free Ammonia Content in Panicles
of Rice Plants

Fig. 16 Effects of Bialaphos, L-PTC, and L-MSO on GS Activity
and Free Ammonia Content in Panicles of Rice Plants

In relationship among application dose of bialaphos, GS activity
and free ammonia content, these results suggest that ammonia
accumulation as a result of GS inhibition directly causes sterility in
rice (Fig. 17).

55
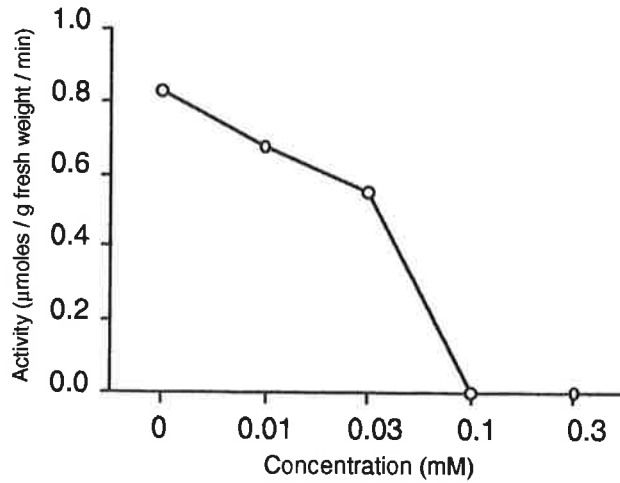
Effect of Bialaphos on Glutamine Synthetase
in Panicles of Rice Plants
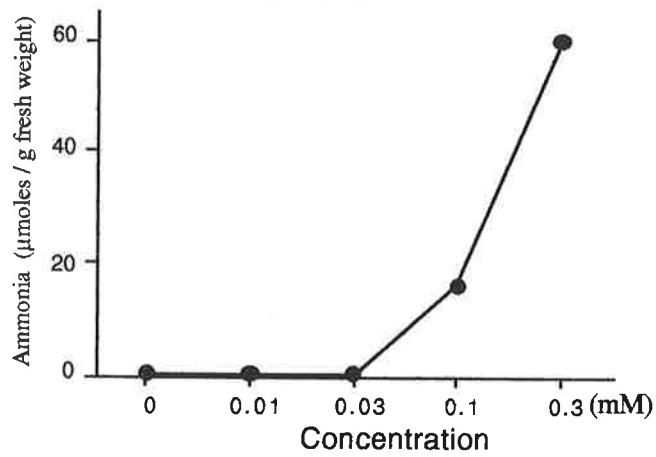
Effect of Bialaphos on Ammonia Content
in Panicles of Rice Plants
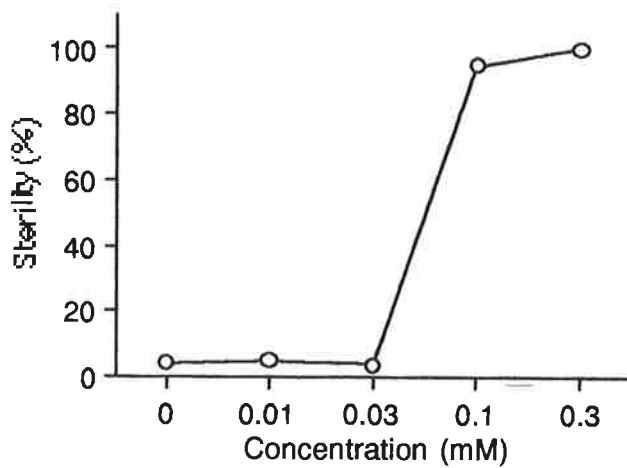
Effect of Bialaphos on Sterility of Rice Plants

Fig. 17 Effects of Bialaphos on GS Activity, Ammonia Content,
and Sterility of Rice Plants

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The decrease of GS activity was observed in the panicles of rice after treatments with 0.1 mM of bialaphos. However, treatment by bialaphos with 100 mM of glutamine did not inhibit GS activity until anthesis, and GS activity in panicles treated with bialaphos and 100 mM of glutamine was similar to that of control (Fig. 18). In according to the inhibition of GS activities, the increases of free ammonia content was observed in the panicles of rice after treatments with 0.1 mM of bialaphos and 1 to 10 mM of glutamine. The accumulation of ammonia was continued until anthesis (Fig. 19). However, treatment by bialaphos and 100 mM of glutamine did not cause the increase of free ammonia content until anthesis.
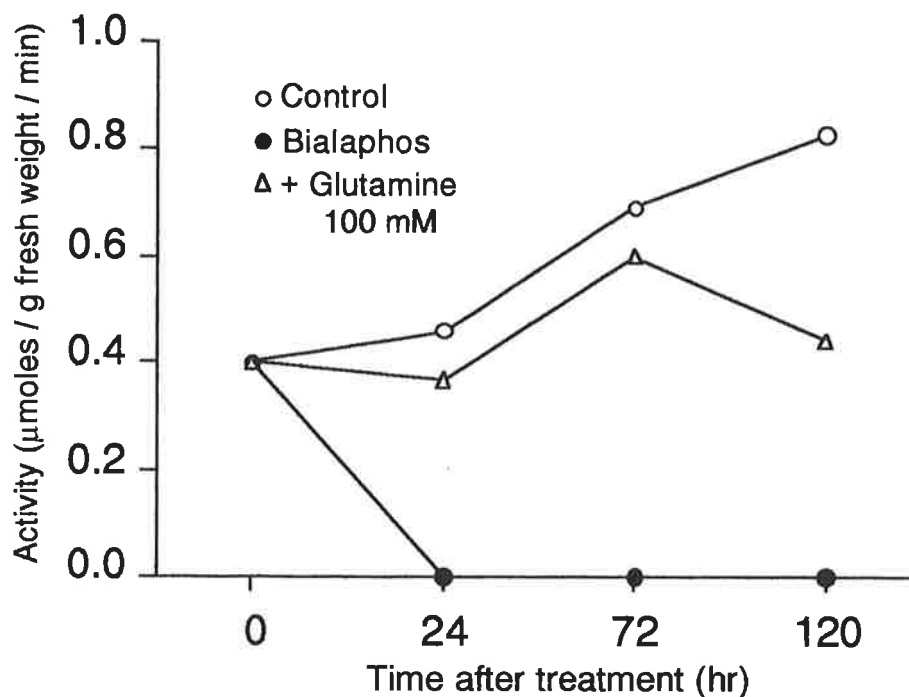
Fig. 18 Effect of Glutamine on Glutamine Synthetase in Panicles of Rice Plants Treated with Bialaphos

57
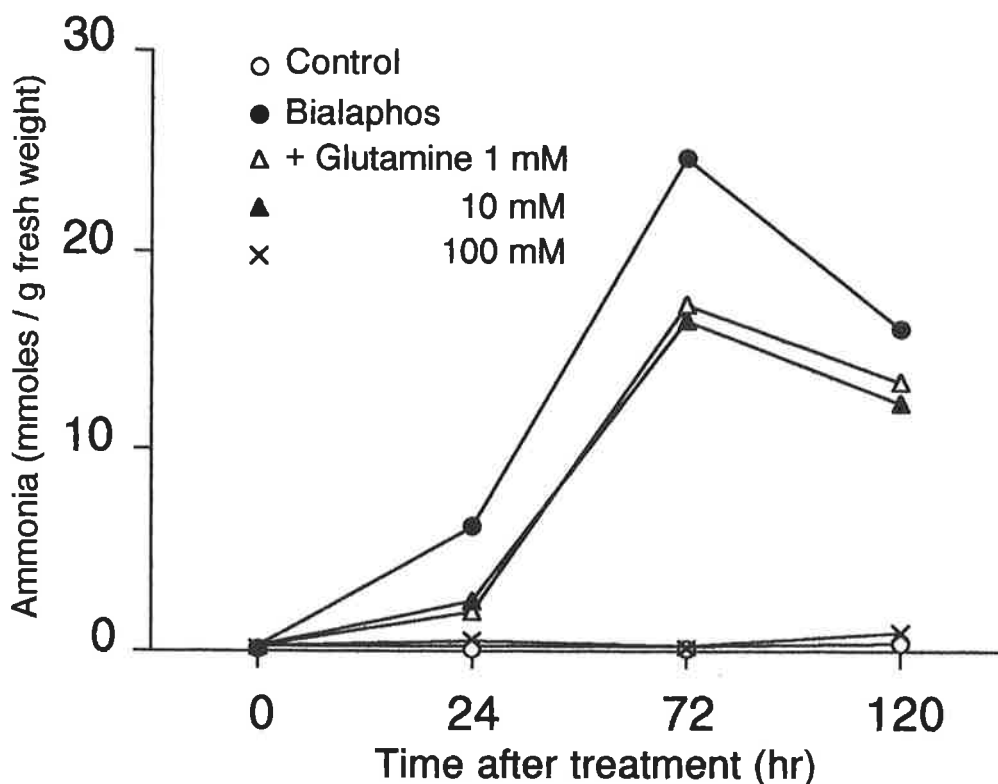
Fig. 19 Effect of Glutamine on Ammonia Content in Panicles of Rice Plants Treated with Bialaphos

GS activities and ammonia contents in panicles treated with ammonia and ammonium sulfate were as the same levels as those of control (Fig. 20). GS activity and ammonia content in panicles treated with bialaphos and DCMU were as the same level as those with bialaphos treatment alone (Fig. 21).

The results *in vivo* and *in vitro* of GS activities treated with bialaphos and L-PTC indicate that L-PTC is the real inhibitor of GS from panicles of rice plants,[45] and that L-PTC is produced by the hydrolysis of bialaphos[42] in the rice plant tissues and inhibits GS activity. Though the exogenous application of glutamine restores GS activity inhibited by bialaphos treatment, the exogenous application dosage of glutamine needs about 1000 times as much as the dosage of bialaphos.
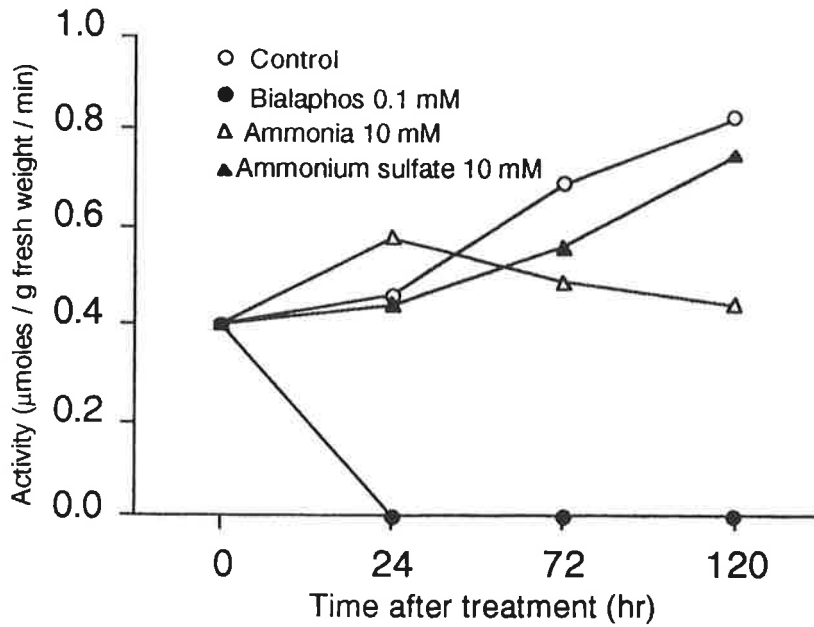
58

Time Course of Glutamine Synthetase Activity
in Panicles of Rice Plants Treated with Bialaphos,
Ammonia and Ammonium sulfate
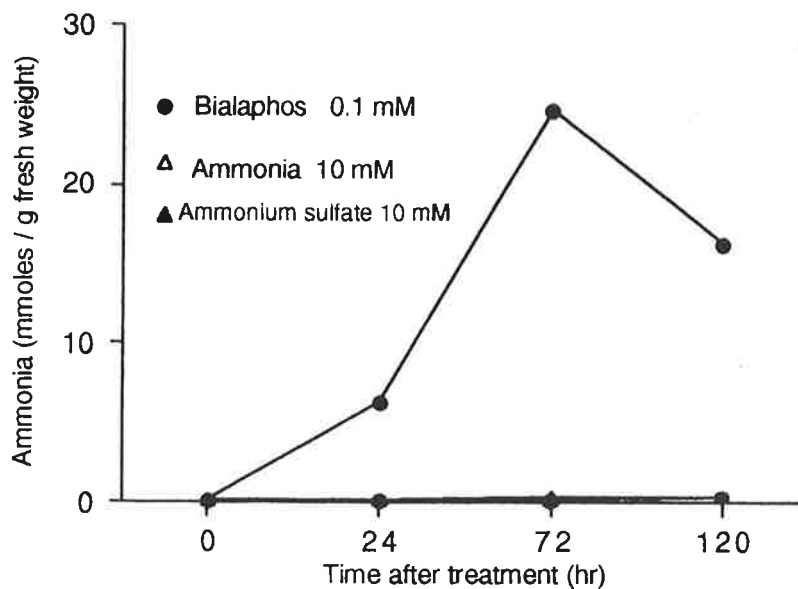
Time Course of Free Ammonia Content in Panicles
of Rice Plants Treated with Bialaphos, Ammonia,
and Ammonium sulfate

Fig. 20 Effects of Ammonia, Ammonium sulfate, and Bialaphos
on GS Activity and Free Ammonia Content in Panicles
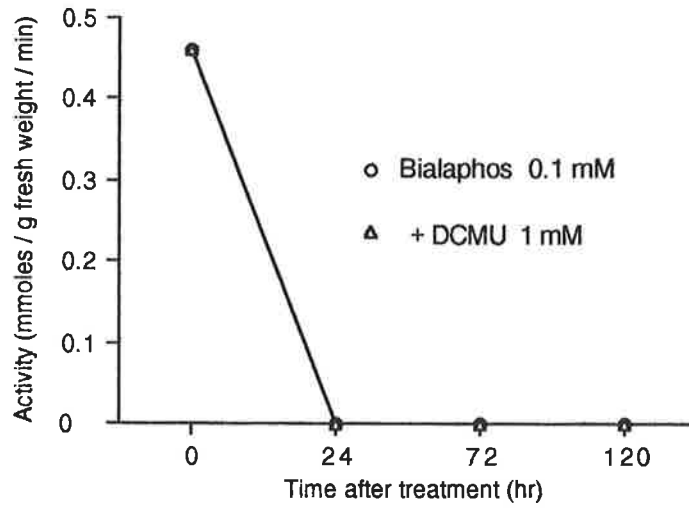
Effect of DCMU on Glutamine Synthetase in Panicles
of Rice Plants Treated with Bialaphos
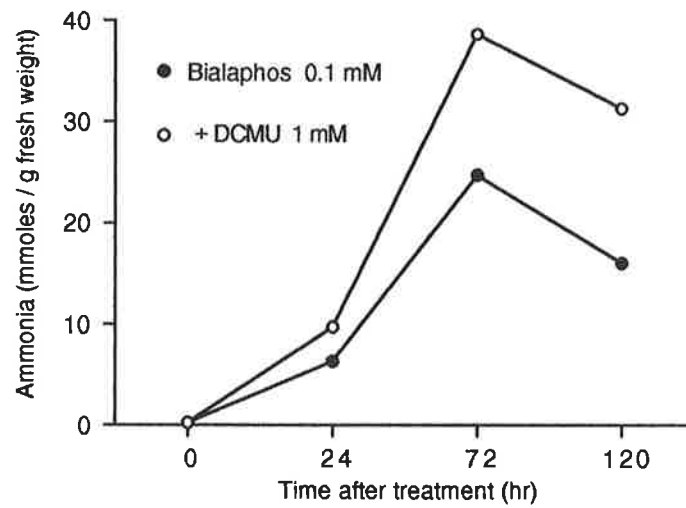
Effect of DCMU on Ammonia Content in Panicles
of Rice Plants Treated with Bialaphos

Fig. 21 Effects of DCMU on GS Activity and Ammonia Content
in Panicles of Rice Plants Treated with Bialaphos

Total and protein nitrogen and free amino acids content in leaves and culms, and panicles

In addition to sterility in rice, effects of accumulation of free ammonia on rice plants were examined.

The decrease of protein nitrogen in panicles was caused by treatment with 0.1 mM of bialaphos and L-PTC (Fig. 22). The same results was also shown in leaves and culms (Fig. 23). On the other hand, the increase of free amino acids content in panicles or leaves and culms was shown by treatment with 0.1 mM of bialaphos and L-PTC (Fig. 24). In particular, the increase was remarkable in panicles. These results suggest that protein synthesis in leaves and panicles is suppressed by the accumulation of ammonia.
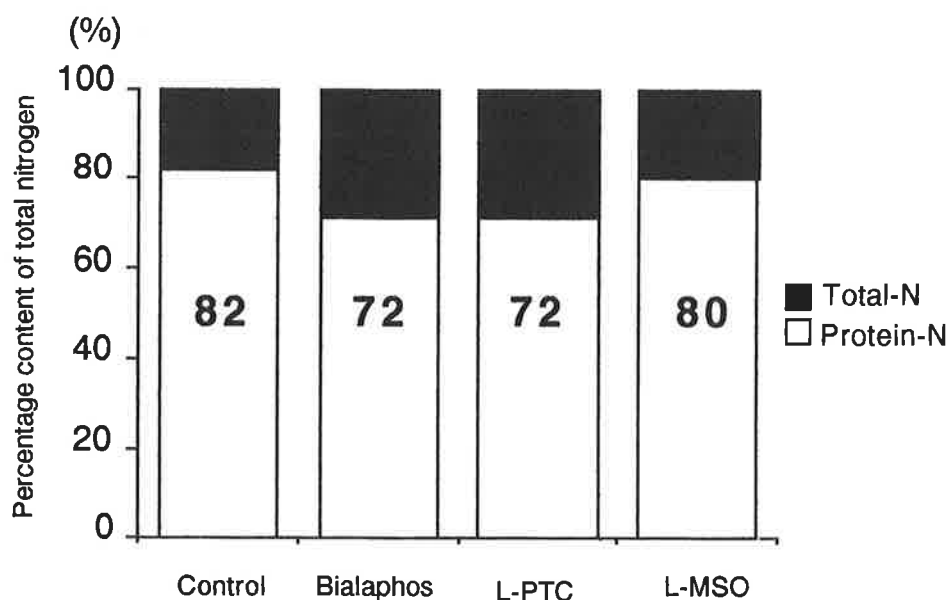
Fig. 22 Effects of Bialaphos, L-PTC and L-MSO
on Nitrogen Content in Panicles of Rice Plants
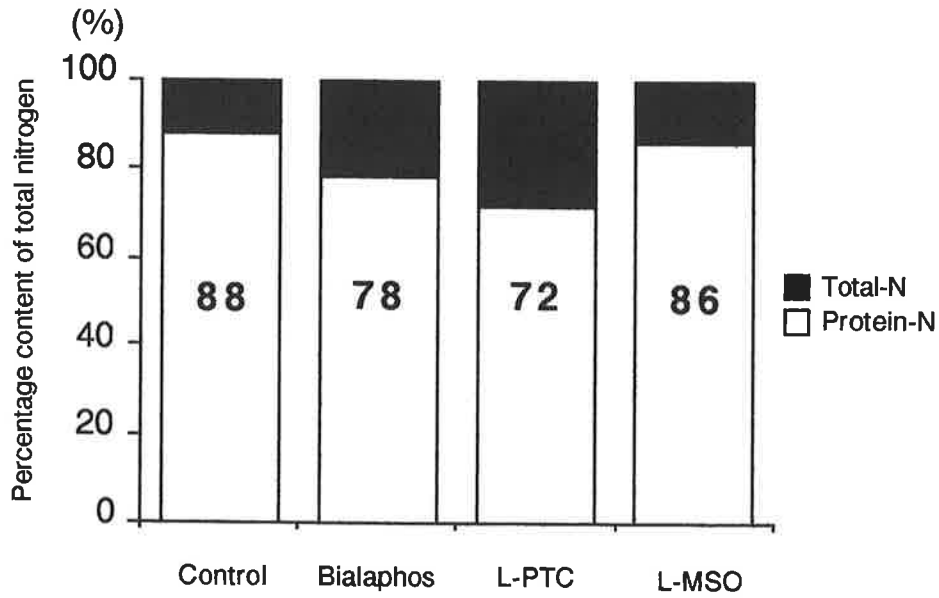
Fig. 23 Effects of Bialaphos, L-PTC and L-MSO on Nitrogen
Content in Leaves and Culms of Rice Plants
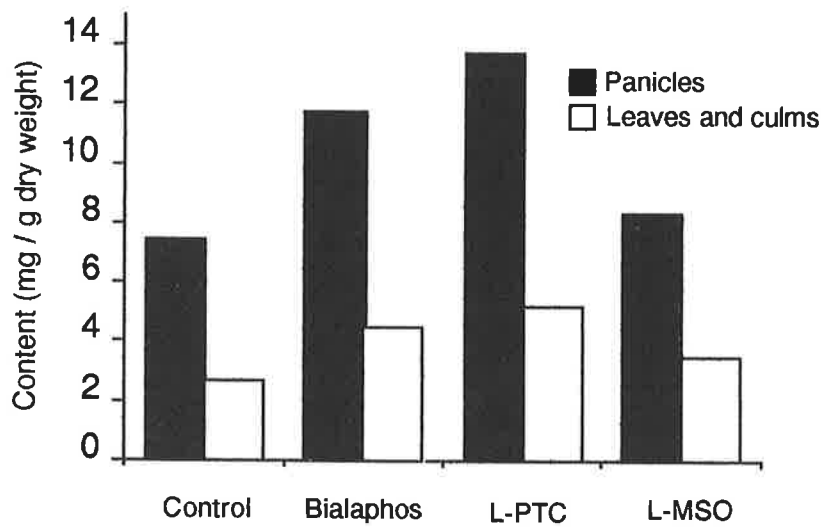
Fig. 24 Effects of Bialaphos, L-PTC and L-MSO on Free
Amino acids in Panicles and, Leaves and Culms
of Rice Plants

The contents of protein nitrogen and free amino acids in leaves and culms, and panicles of rice plants treated with bialaphos and 100 mM of glutamine were the same level as those of control (Fig. 25). In the same way, the contents of protein nitrogen and free amino acids in leaves and culms, and panicles of rice plants treated with ammonia were as the same level as those of control (Fig. 26).

The contents of protein nitrogen and free amino acids in leaves and culms, and panicles of rice plants treated with bialaphos and DCMU were as the same level as those with bialaphos treatment alone (Fig. 27).

Since GS is the first enzyme in the primary pathway of ammonium assimilation, the change of GS activity affects protein synthesis in the tissues.[69,70] The results of protein nitrogen and free amino acids content in this investigation indicate that protein synthesis is reduced by the ammonia accumulation as a result of GS inhibition, and suggest that glutamine is the major molecular in the protein synthesis because of the restoration of protein nitrogen and GS activity.
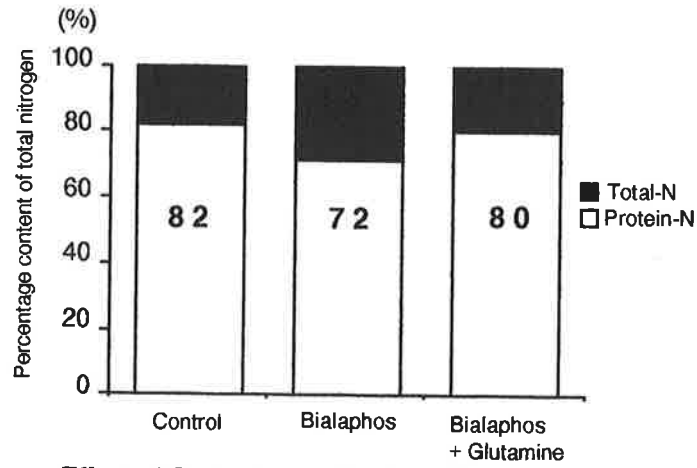
(%)

Effect of Glutamine on Nitrogen Content in Panicles
of Rice Plants Treated with Bialaphos

(%)
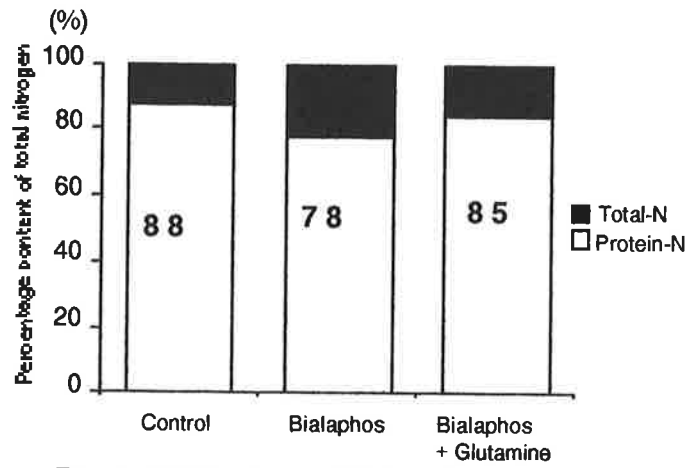
Effect of Glutamine on Nitrogen Content in Leaves
and Culms of Rice Plants Treated with Bialaphos
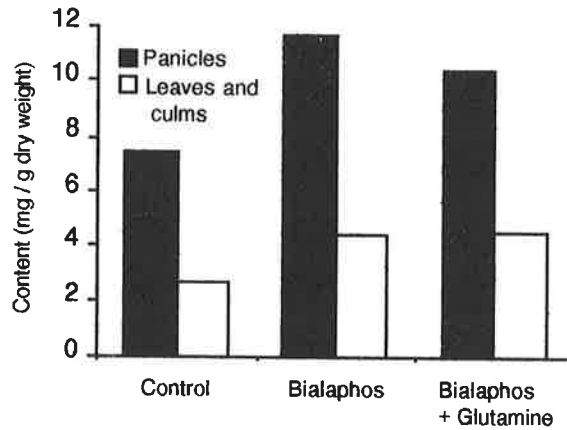
Effect of Glutamine on Free Amino acids in Panicles and,
Leaves and Culms of Rice Plants Treated with Bialaphos

Fig. 25 Effects of Glutamine on Nitrogen Content and Free
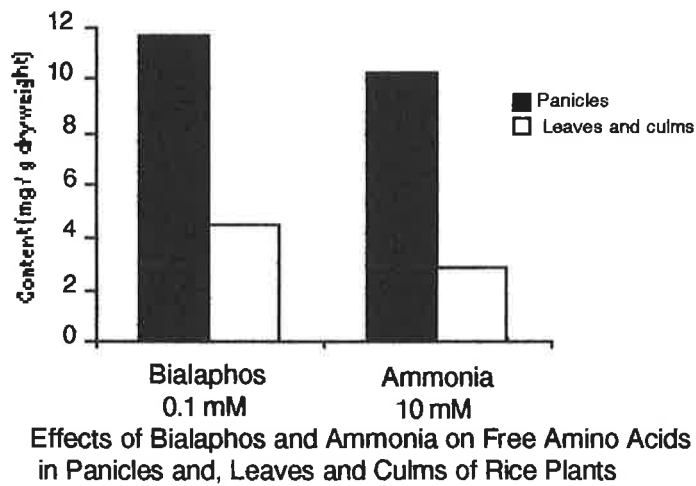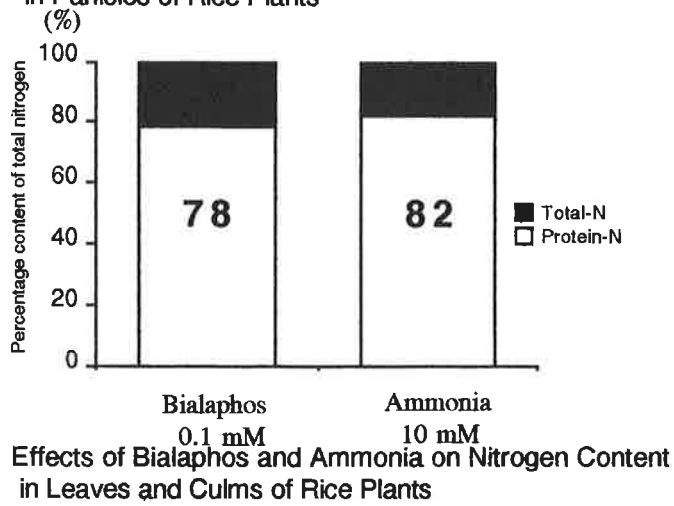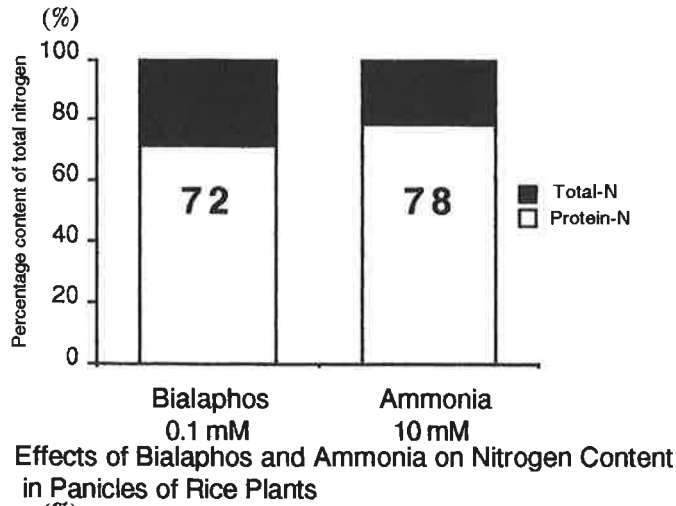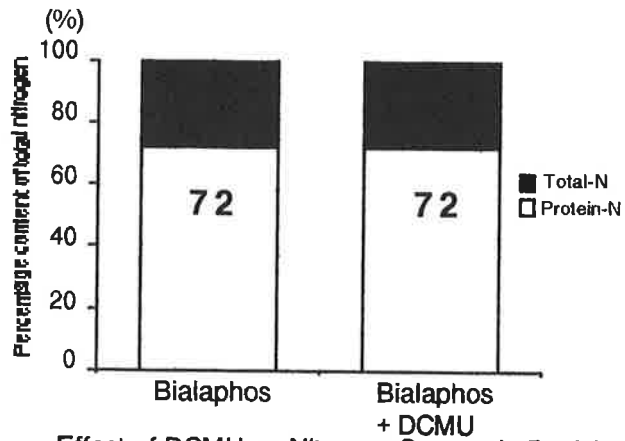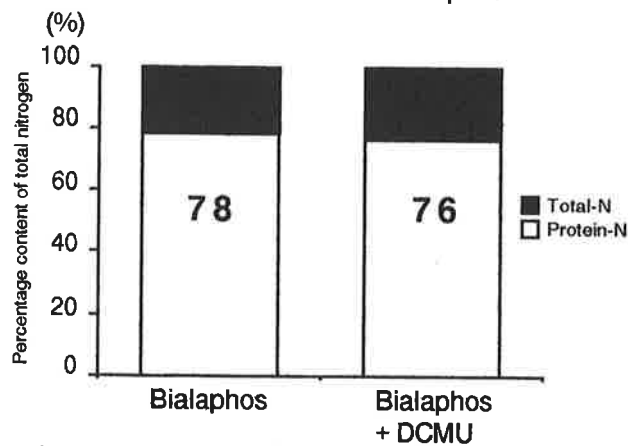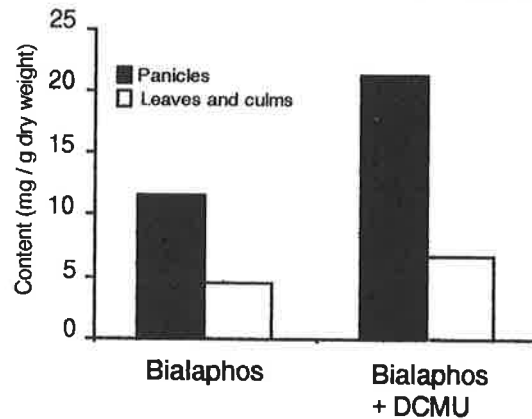Amino acids Content in Panicles, Leaves and Culms of Rice Plants
Treated with Bialaphos

Effects of Bialaphos and Ammonia on Nitrogen Content
in Panicles of Rice Plants

Effects of Bialaphos and Ammonia on Nitrogen Content
in Leaves and Culms of Rice Plants

Effects of Bialaphos and Ammonia on Free Amino Acids
in Panicles and, Leaves and Culms of Rice Plants

Fig. 26 Effects of Bialaphos and Ammonia on Nitrogen Content
and Free Amino acids Content in Panicles, Leaves and Culms of
Rice Plants

6 5

Effect of DCMU on Nitrogen Content in Panicles
of Rice Plants Treated with Bialaphos

Effect of DCMU on Nitrogen Content in Leaves and
Culms of Rice Plants Treated with Bialaphos

Effect of DCMU on Free Amino acids in Panicles and,
Leaves and Culms of Rice Plants Treated with Bialaphos

Fig. 27 Effects of DCMU on Nitrogen Content and
Free Amino acids Content in Panicles, Leaves and
Culms of Rice Plants Treated with Bialaphos

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Changes of free amino acids in anthers

The total contents of free amino acids in untreated anthers and in anthers by the treatments of bialaphos and bialaphos with glutamine were 17.20 nM, 1.69 nM and 22.67 nM per anther, respectively. The rate of proline was about a half of free amino acids in untreated anthers. Although the role of proline in plants were known to be utilized in new protein synthesis[164] and used as an energy source,[165] the role played by proline content was not clearly understood in connection with pollen fertility.[131,166]

Table 13. Free Amino acids Composition (%) of Anther

Amino acids	Control	Bialaphos 0.1 mM	Bialaphos 0.1 mM + Glutamine 100 mM
Aspartic acid	2.02	5.74	1.82
Threonine	5.63	22.56	6.48
Serine	6.28	9.61	5.94
Glutamic acid	2.51	7.92	2.30
Proline	51.04	15.14	33.45
Glycine	2.12	5.28	2.24
Alanine	18.21	8.66	32.32
Cysteine	0.24	0.26	0.36
Valine	2.77	5.98	4.16
Methionine	0.29	0.14	0.42
Isoleucine	0.45	1.15	0.86
Leucine	0.75	1.85	1.52
Tyrosine	1.18	0.67	1.02
Phenylalanine	0.82	2.78	1.53
Histidine	1.73	8.50	1.74
Lysine	1.89	1.87	1.57
Arginine	2.07	1.89	2.27

The untreated anthers had about 3.4 times more proline than the anthers treated with bialaphos, while the treated anthers had about 3 times more aspartic acid and glutamic acid than the untreated anthers. Furthermore, the treated anthers had about 4 times more threonine than the untreated anthers (Table 13). The anthers which had been restored fertility by treatment with bialaphos and glutamine exhibited the increases of proline. The other free amino acids of the anthers treated with bialaphos and glutamine were similar to those of the untreated anthers(Table 13). The exogeneous application of glutamine restored normal amino acid metabolism.

The decrease of proline caused in anthers by bialaphos treatment was similar to that of such cytoplasmic male sterile anthers as rice,[129,130] wheat, maize,[131] sorghum[132] and sugar beet.[133] The characteristic changes of free amino acids in anthers treated with bialaphos were the decreases of proline and the increase of glutamic acid, glutamic acid, and threonine. Bialaphos, the inhibitor of GS, inhibits the conversion of glutamic acid to glutamine.[45] As a result of glutamine deficiency, the conversion of aspartic acid to asparagine is inhibited because glutamine plays an important role as the amide nitrogen donor in asparagine synthesis in rice plants.[167,168] Proline synthesis is also inhibited, since asparagine is an precoursor of proline.[169,170] Furthermore, accumulated aspartic acid metabolites into threonine, because aspartic acid is an precoursor of threonine. Therefore, the decrease of proline and the increase of glutamic acid , aspartic acid and threonine which occur in sterile anthers treated with bialaphos is easily explained (Fig. 28).
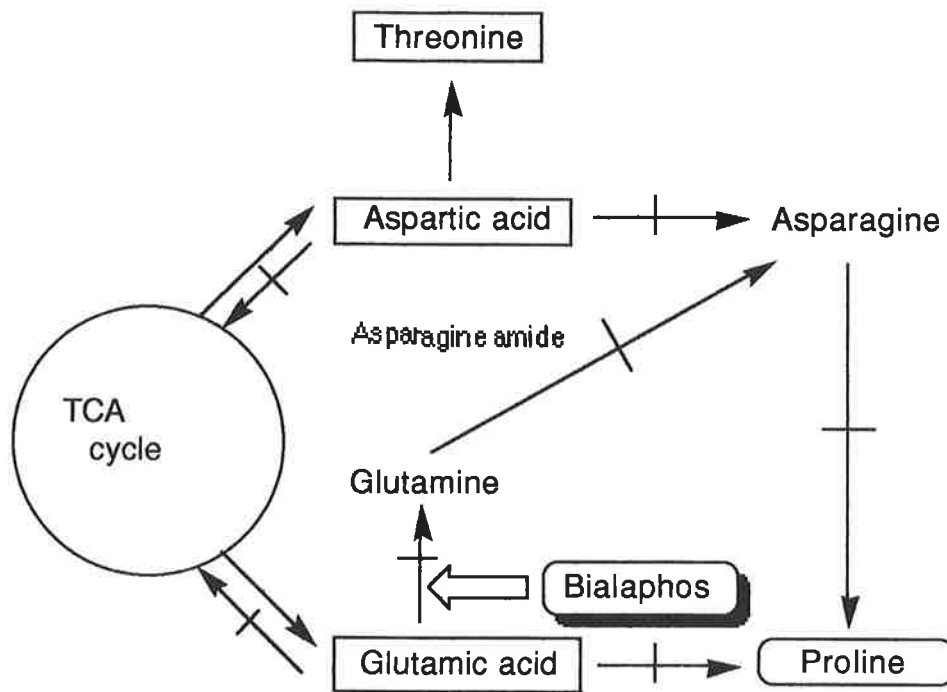
Fig. 28 The Possible Pathway of Abnormal Amino acid Metabolism
in the Sterile Anthers Treated with Bialaphos

☐ : Accumulation, ⬭ : Decrease

In the free amino acids composition of leaves and culms (Table 14), the rate of alanine was about a quarter of free amino acids in the treated leaves and culms. The high proline content is the character in the anthers in rice plants, because the rate of proline in leaves and culms is much lower than that in anthers. Since the increase of threonine is shown in leaves and culms in the both treatments, the increase of threonine in leaves and culms does not relate to rice sterility. The other free amino acids do not have any changes between the untreated leaves and culms and those treated with bialaphos.

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Table 14. Free Amino acids Composition (%) of Leaves and Culms

Amino acids	Control	Bialaphos 0.1 mM	Bialaphos 0.1 mM + Glutamine 100 mM
Aspartic acid	2.91	5.90	4.54
Threonine	4.46	10.14	12.77
Serine	5.28	4.44	5.26
Glutamic acid	6.31	5.14	6.67
Proline	13.00	9.47	9.59
Glycine	3.73	2.40	2.49
Alanine	28.39	20.92	24.94
Cysteine	1.40	tr.	tr.
Valine	7.71	11.48	10.09
Methionine	tr.	tr.	tr.
Isoleucine	1.23	2.02	2.21
Leucine	1.28	1.37	1.41
Tyrosine	12.36	12.55	13.86
Phenylalanine	2.26	2.49	2.67
Histidine	4.39	6.41	tr.
Lysine	1.12	tr.	0.70
Arginine	4.17	5.27	3.32

tr. : Trace

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Changes of the component of carbohydrates in leaves and culms, and panicles

In order to know the effects of ammonia accumulation on carbohydrate metabolism in leaves and culms, and panicles, the component of various carbohydrates in those which were immediately before anthesis were examined by the analytical method of carbohydrates.

Figure 29 shows the component of various carbohydrates such as the reducing sugar, non-reducing sugar, starch and hemicellulose in panicles treated with three GS inhibitors and an untreated panicles. The non-reducing sugar in panicles treated with 0.1 mM of bialaphos and L-PTC increased about 2 times as much as that of control. However, the non-reducing sugar in panicles treated with 0.1 mM of L-MSO was the same as that of control. The same results were shown in the compornent of various carbohydrates of leaves and culms (Fig. 30).
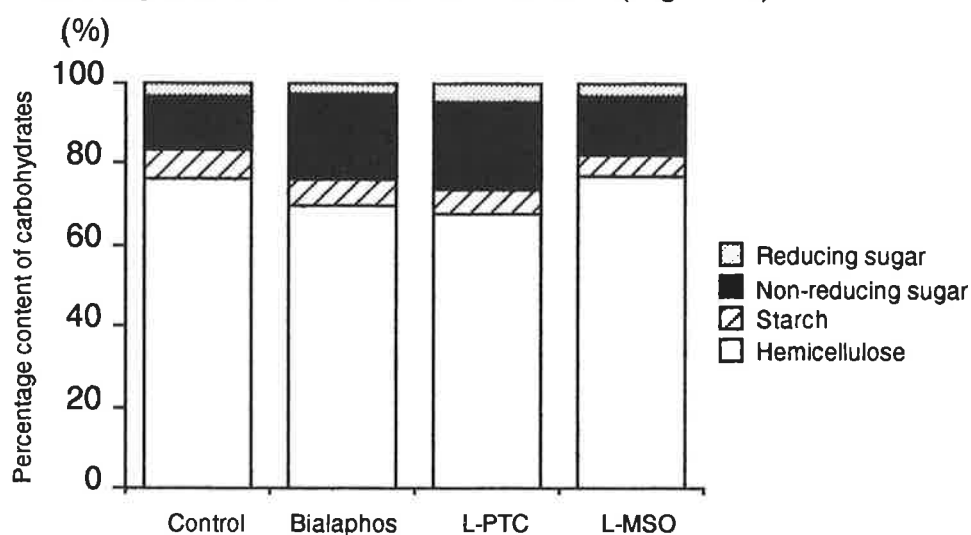
Fig. 29 Effects of Bialaphos, L-PTC and L-MSO on Various Carbohydrates in Panicles of Rice Plants
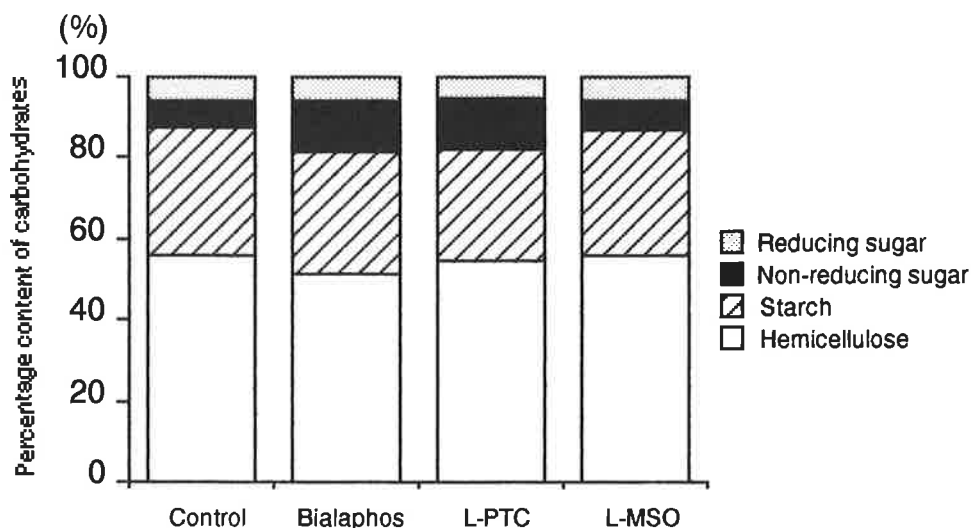
Fig. 30 Effects of Bialaphos, L-PTC and L-MSO on Various Carbohydrates in Leaves and Culms of Rice Plants

The components of various carbohydrates in leaves and culms, and panicles of rice plants treated with bialaphos and 100 mM of glutamine were the same level as those of control (Fig. 31). In the same way, the components of various carbohydrates in leaves and culms, and panicles of rice plants treated with ammonia were as the same level as those of control (Fig. 32). However, the components of various carbohydrates in leaves and culms, and panicles of rice plants treated with bialaphos and DCMU were as the same level as those with bialaphos treatment alone (Fig. 33).

The results of the components of various carbohydrates by the ammonium accumulation indicate that the increase of the non-reducing sugar is one of the characteristics of the ammonium accumulation.

According to the paper chromatography, it was estimated that the non-reducing sugar in tissues of all treatments was only sucrose and

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the reducing sugars were glucose and fructose. It was known that the form of sugar translocated from leaves to other parts of rice plant was sucrose, and that sucrose was hydrolyzed to reducing sugars and starch was biosynthesized from reducing sugars by phospholylated process in such parts as leaf, culm and panicle.[80] Therefore, the accumulation of the non-reducing sugar by treatment with bialaphos and L-PTC affected the carbohydrate matebolism of panicle, leaf and culm of the rice plant.
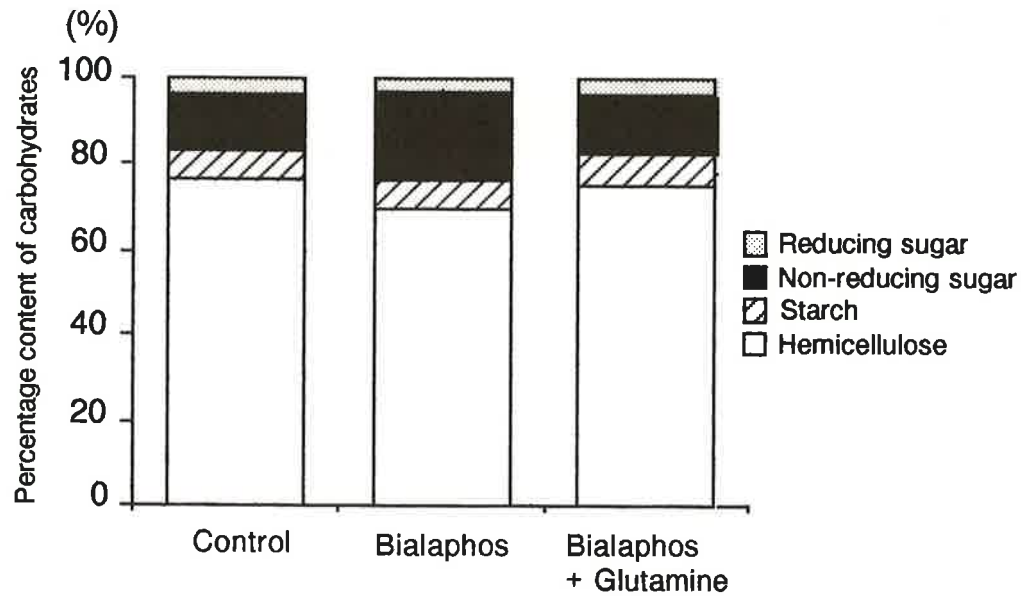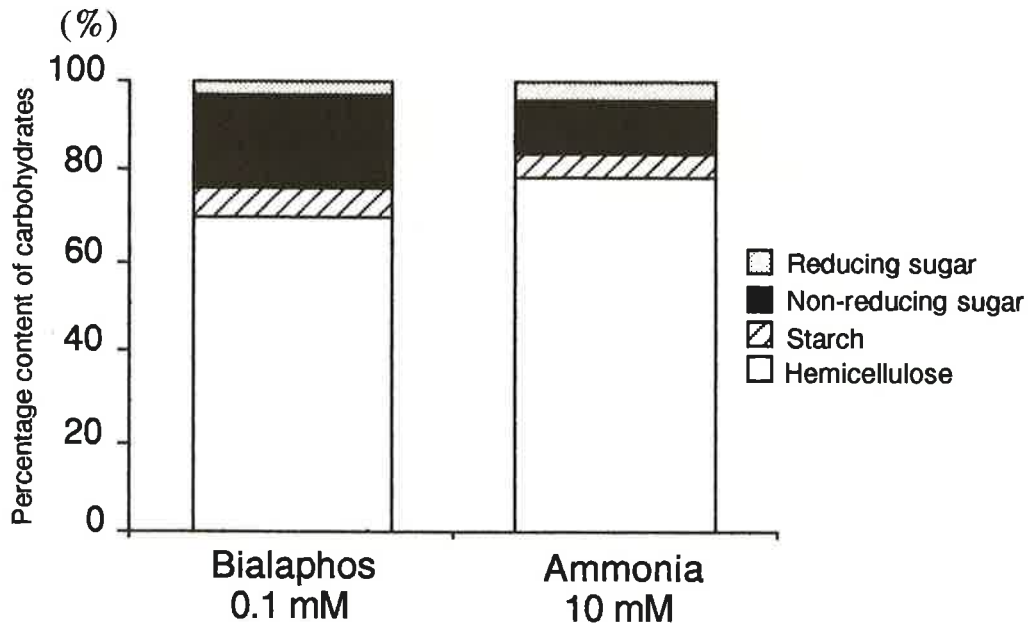
Effect of Glutamine on Various Carbohydrates in Panicles of Rice Plants Treated with Bialaphos
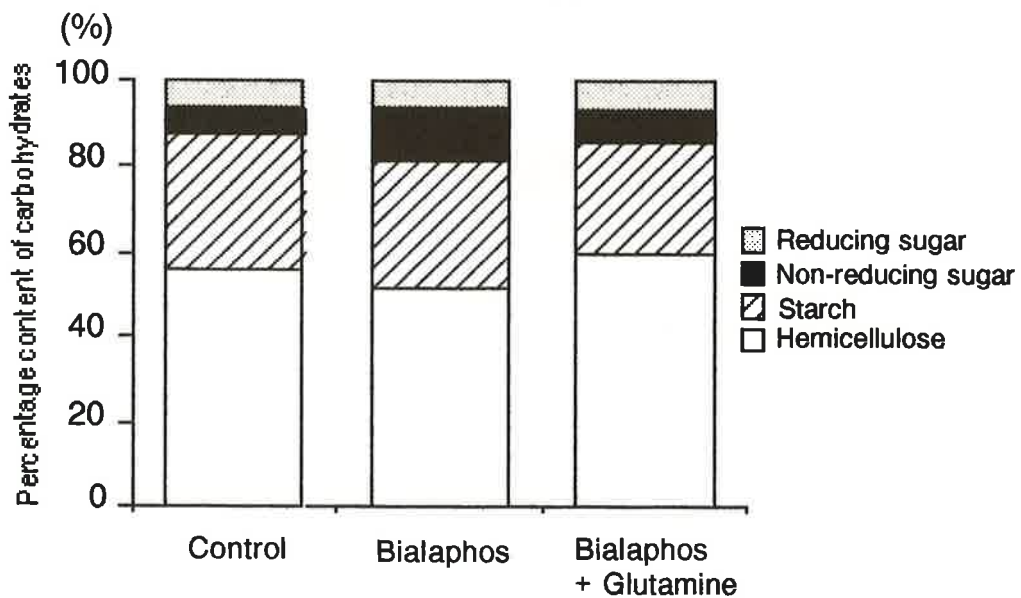
Effect of Glutamine on Various Carbohydrates in Leaves and Culms of Rice Plants Treated with Bialaphos

Fig. 31 Effects of Glutamine on Various Carbohydrates in Panicles, Leaves and Culms of Rice Plants Treated with Bialaphos
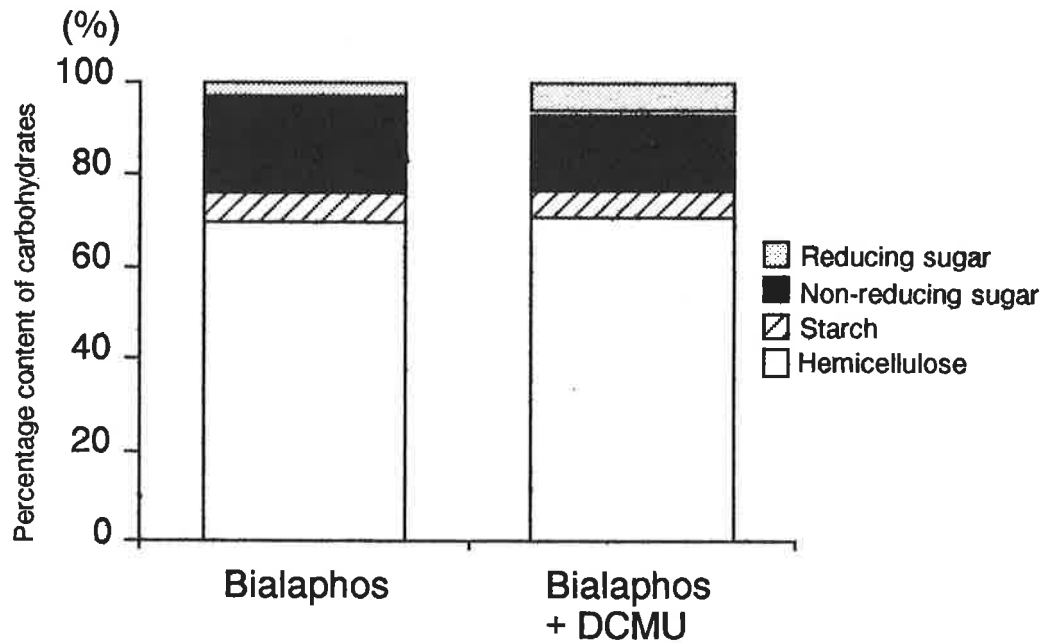
Effects of Bialaphos and Ammonia on Various Carbohydrates in Panicles of Rice Plants
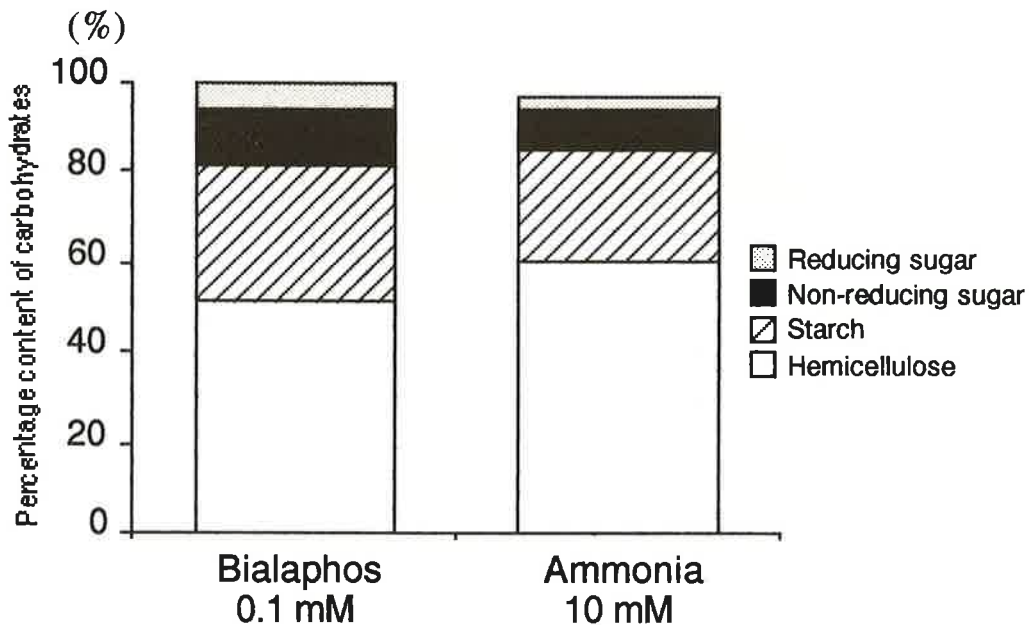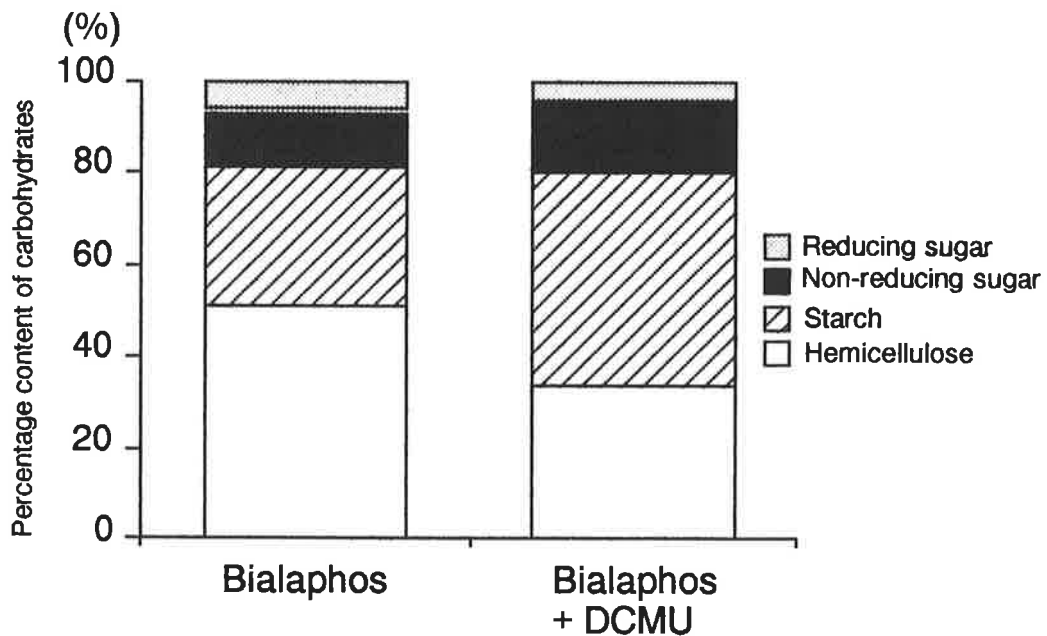

Effects of Bialaphos and Ammonia on Various Carbohydrates in Leaves and Culms of Rice Plants

Fig. 32 Effects of Bialaphos and Ammonia on Various Carbohydrates in Panicles, Leaves and Culms of Rice Plants

(%)

Percentage content of carbohydrates

100

80

60

40

20

0

Bialaphos　　Bialaphos + DCMU

Reducing sugar
Non-reducing sugar
Starch
Hemicellulose

Effect of DCMU on Various Carbohydrates in Panicles
of Rice Plants Treated with Bialaphos

(%)

Percentage content of carbohydrates

100

80

60

40

20

0

Bialaphos　　Bialaphos + DCMU

Reducing sugar
Non-reducing sugar
Starch
Hemicellulose

Effect of DCMU on Various Carbohydrates in Leaves
and Culms of Rice Plants Treated with Bialaphos

Fig. 33 Effects of DCMU on Various Carbohydrates in Panicles,
Leaves and Culms of Rice Plants Treated with Bialaphos

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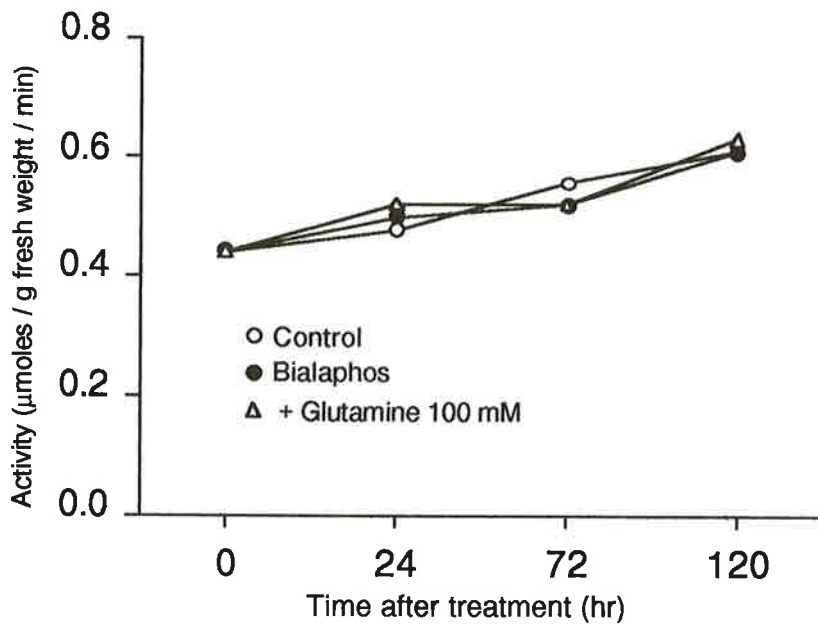*ATPase activity in panicles of the sterile rice plants*

In order to know the viability of panicles in rice, ATPase activities in panicles treated with GS inhibitors were examined until anthesis. ATPase activities in panicles treated with 0.1 mM of bialaphos, L-PTC and L-MSO were the same as that of control, respectively. It was suggested that the viability of panicles treated with GS inhibitors was persistent until anthesis (Fig. 34).

ATPase activity in panicles treated with bialaphos and 100 mM of glutamine were the same level as those of control (Fig. 34). ATPase activity in panicles treated with ammonia and ammonium sulfate were the same level as those of control (Fig. 35). ATPase activity in panicles treated with bialaphos and DCMU were as the same level as those with bialaphos treatment alone (Fig. 35).

The results of the ATPase activities in this investigation indicate that the ATPase activities in panicles of all treatments except orthovanadate are the same as that in the untreated panicles. The maintenance of the ATPase activity may be one of the evidence of the viability in panicles.
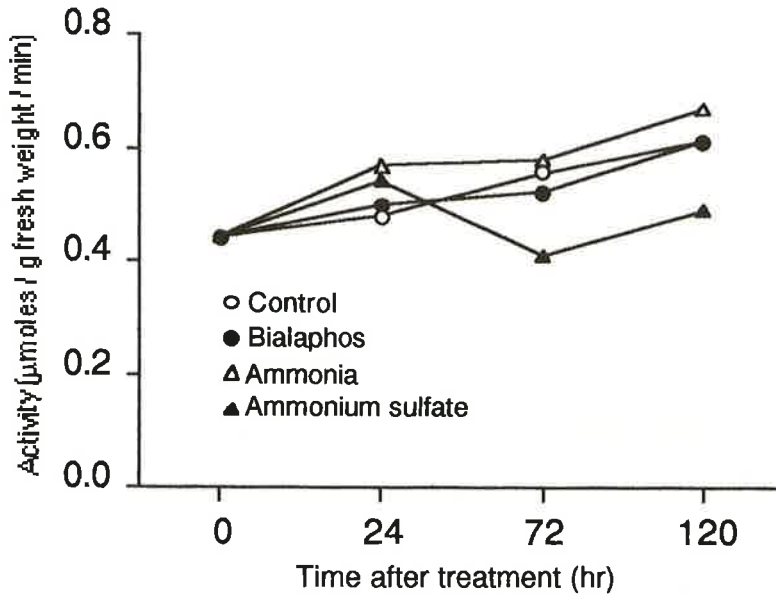
77
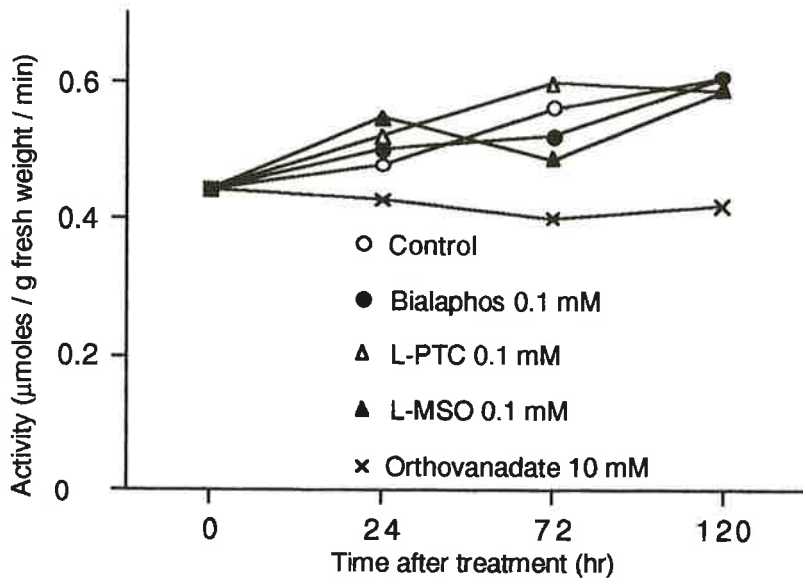
Effects of Bialaphos, L-PTC, L-MSO and Orthovanadate
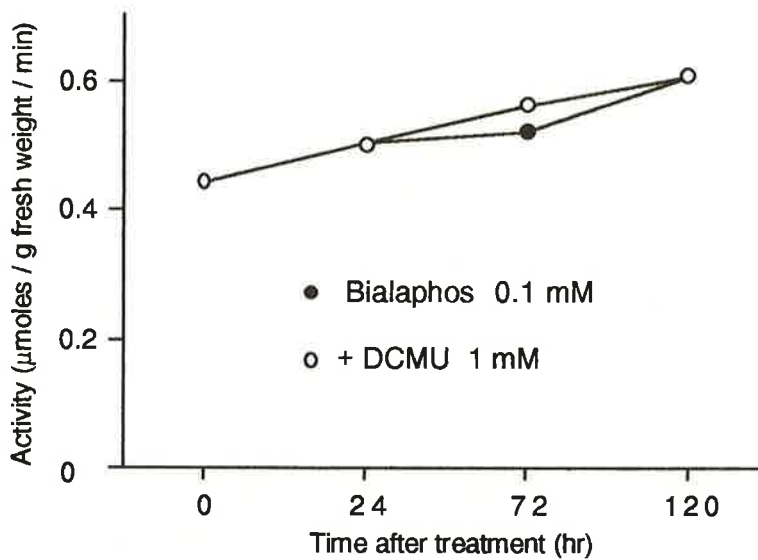on ATPase Activity in Panicles of Rice Plants

Effect of Glutamine on ATPase Activity in Panicles
of Rice Plants Treated with Bialaphos

Fig. 34 Effects of Bialaphos, L-PTC, L-MSO, Orthovanadate,
and Glutamine on ATPase Activity in Panicles of Rice Plants

Time Course of ATPase Activity in Panicles of Rice
Plants Treated with Bialaphos, Ammonia and
Ammonium sulfate

Effect of DCMU on ATPase activity in Panicles
of Rice Plants Treated with Bialaphos

Fig. 35 Effects of Bialaphos, Ammonia, Ammonium sulfate,
and DCMU on ATPase Activity in Panicles of Rice Plants

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Changes of chlorophyll in leaves of the sterile rice plants

Since the decrease of chlorophyll is known as the phytotoxicity of ammonia in rice[182] and tomato,[183] the changes of chlorophyll in leaves of rice treated with GS inhibitors were examined until maturation period. Since chlorophyll content in leaves extracted by 80 % ethanol and the SPAD value measured by chlorophyll meter (SPAD-502, MINOLTA CO.) gave a high coefficient of correlation (Fig. 36), the SPAD value was used thereafter because of its rapid and high sensitivity in the assay.
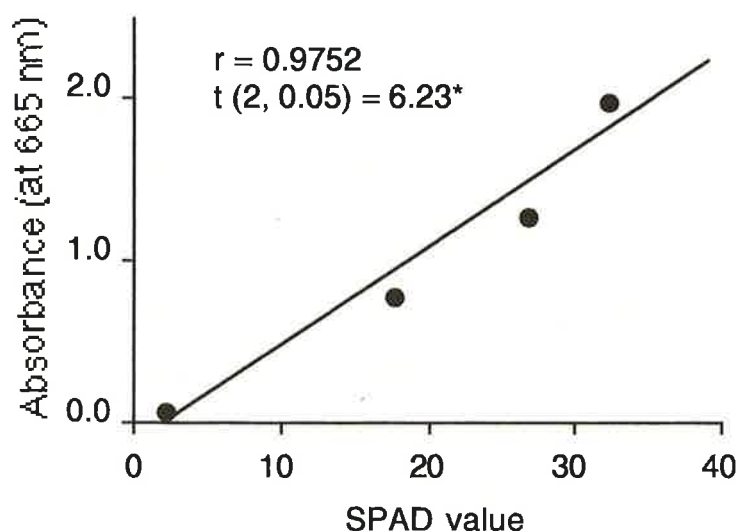

Fig. 36 Corelationship between SPAD Value
and Chlorophill Concentration

The decreases of chlorophyll in leaves treated with bialaphos at the concentrations of 0.01, 0.03 and 0.1 mM indicated as the same tendency as that of control, but the remarked decrease of chlorophyll in leaves treated at a concentration of 0.3 mM was observed from 7 days after treatment. In the treatment of L-PTC (0.01-0.3 mM), the same decreases of chlorophyll in leaves were observed until maturation period (Fig. 37).

The decreases of chlorophyll in leaves treated with L-MSO at the concentrations of 0.01 to 0.3 mM indicated as the same tendency as that of control until maturation period (Fig. 37).

These results indicate that the noticeable decreases of chlorophyll in leaves treated with bialaphos and L-PTC as a result of the ammonia accumulation are observed only at a concentration of 0.3 mM.

The decrease of chlorophyll in leaves treated with bialaphos and 100 mM of glutamine was the same level as those of control (Fig. 38). Furthermore, the decrease of chlorophyll in leaves treated with ammonia and ammonium sulfate were also the same levels as those of control (Fig. 38). The decrease of chlorophyll in leaves treated with bialaphos and DCMU indicated as the same tendency as those with bialaphos treatment alone (Fig. 38).

One of the characteristics of the ammonia accumulation is the yellowing of the leaves.[182,183] The results of the chlorophyll determinations in this investigation indicate that the chlorophyll content of the leaves was reduced very early after the intiation of GS inhibitor's treatment. The rapid chlorophyll loss may be explained by an effect of the ammonia accumulation on the biosynthesis of chlorophyll.[184] Furthermore, photosynthesis is restricted by the ammonia accumulation. The concentration of ammonia in the leaves was sufficiently high to uncouple photophosphorylation and to inhibit NADP reduction.[185,186] Reduced photophosphorylation may have lowered the energy available for protein synthesis and restricted protein synthesis.[183]
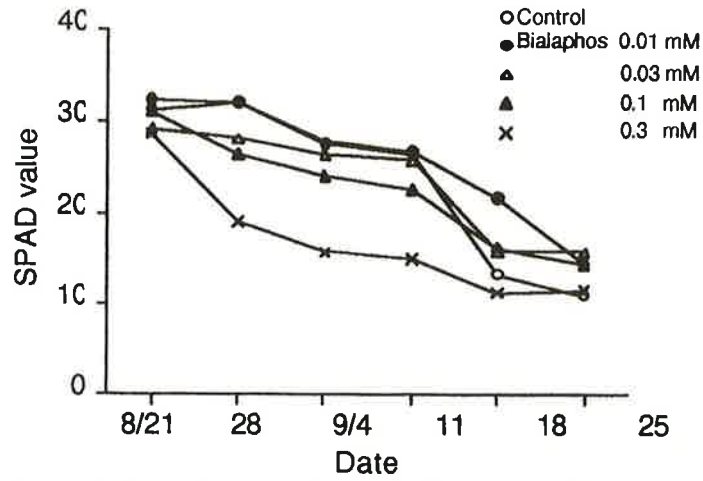
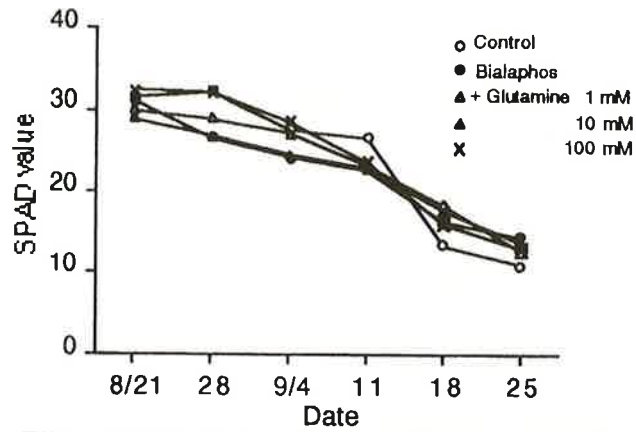Effect of Bialaphos on Chlorophyll in Leaves of Rice Plants
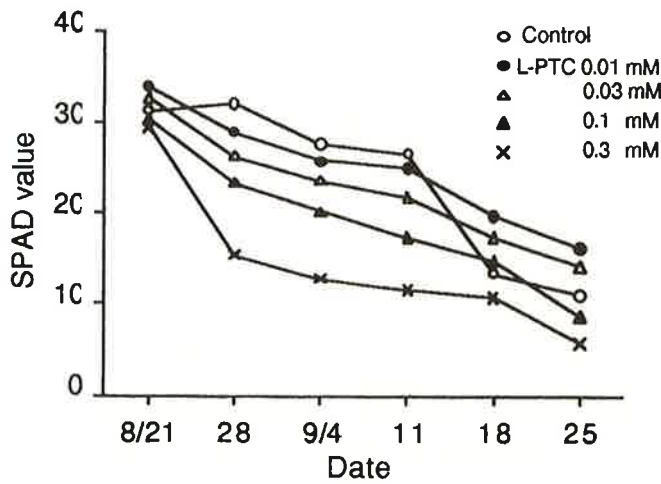
Effect of L-PTC on Chlorophyll in Leaves of Rice Plants
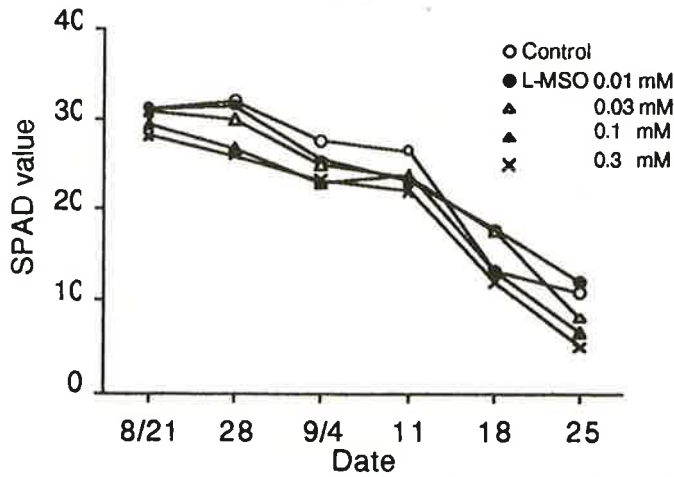
Effect of L-MSO on Chlorophyll in Leaves of Rice Plants

Fig. 37 Effects of Bialaphos, L-PTC and L-MSO
on Chlorophyll in Leaves of Rice Plants

8 2

Effect of Glutamine on Chlorophyll in Leaves of Rice
Plants Treated with Bialaphos
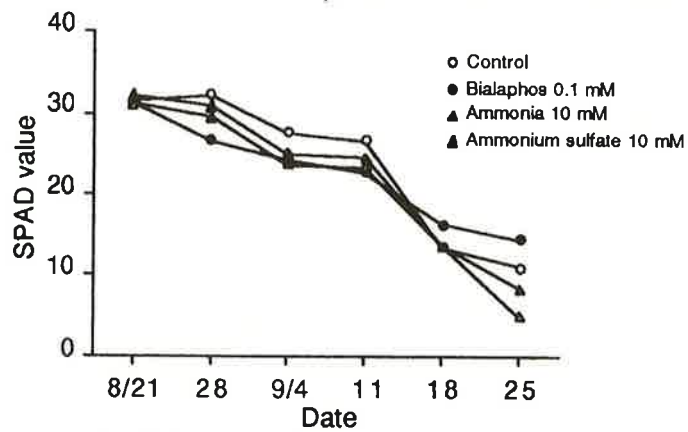
Effects of Bialaphos, Ammonia and Ammonium sulfate
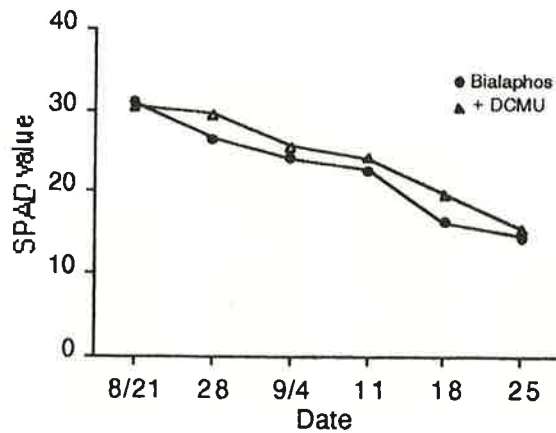on Chlorophyll in Leaves of Rice Plants

Effect of DCMU on Chlorophyll in Leaves of Rice
Plants Treated with Bialaphos

Fig. 38 Effects of Chemicals on Chlorophyll in Leaves
of Rice Plants

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Effects of GS inhibitors on root activity in rice

The root activities of rice plants treated with GS inhibitors were examined before harvesting. Bialaphos, L-PTC and L-MSO exhibited the root activity of 82 %, 106 % and 66 % at a concentration of 0.1 mM, respectively. Furthermore, bialaphos exhibited the root activity of 128 % at a concentration of 0.3 mM (Fig. 39). These results suggest that the high root activity is caused by the complete sterility in rice.

It is very important to know the root activity of rice plants from the standpoint of the agronomical technique, and this activity has a high correlation to the respiratory activity of rice plants.[87] Since the accumulation of nutrients is not caused in panicles by the complete sterility of the panicles in rice plants, the nutrients may be used for the root respiration, and the high root activity may be caused in the complete sterile rice plants.
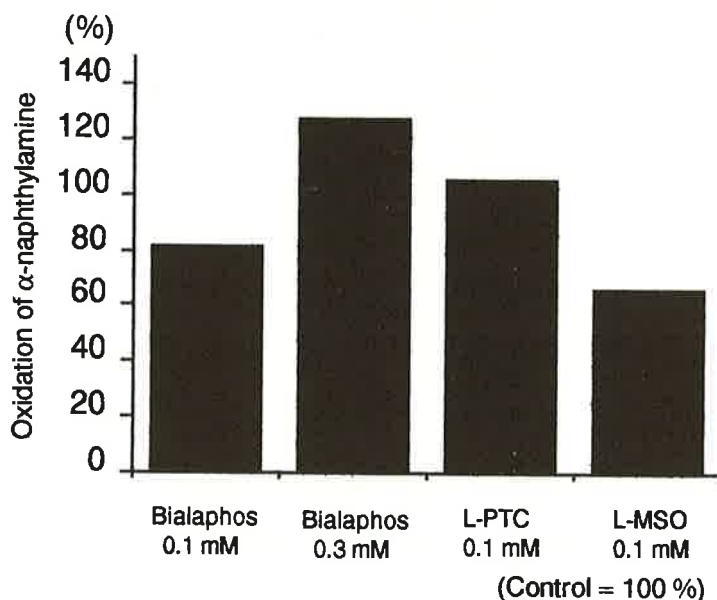
Fig. 39 Effects of Bialaphos, L-PTC and L-MSO
on the Root Activity of Rice Plants

Rice sterility induced by S35-C2

Screening for GS inhibitor from actinomycetes

Since the high sterility in rice is caused by GS inhibitors such as bialaphos and L-PTC, screening for GS inhibitor was tested by using culture filtrates from actinomycetes. Of about 600 actinomycetes isolated, 5 strains were found to produce GS inhibitors in their culture filtrate. Since the extract of culture filtrate from strain S35 was the highest yield of those of 5 strains, S35 was chosen and used further experiments (Table 15).

Table 15. Yield of Metabolites Obtained from Actinomycetes

Strain	Yield (g/l)
S- 35	2.22
S-139	1.98
S-188	1.36
S-429	1.66
S-577	0.88

Taxonomy of strain S35

Strain S35 was cultivated at 30 °C for 14 days on various media such as yeast extract - malt extract agar, glucose - asparagine agar and tyrosine agar shown in Table16. Its morphological characteristics were examined with both optical and electron microscopes. The aerial mycelia were simply branched and formed flexible chains of spores with about 10 spores per chain (0.6 X 1.0 μm) (Fig. 40).
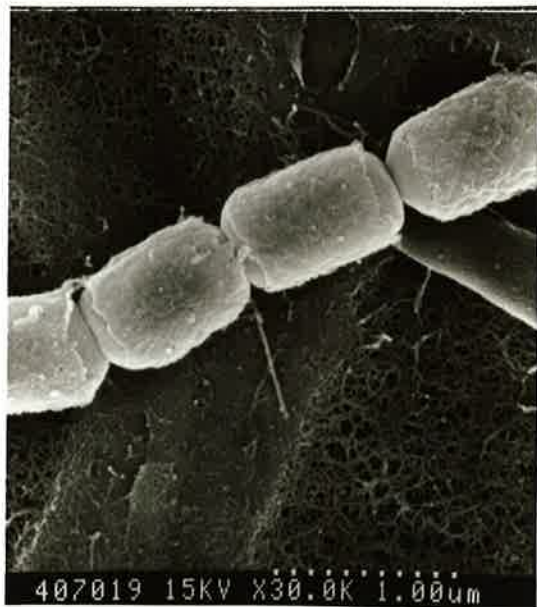
85

Fig. 40 Electron Micrographs of Strain S-35

The spore surface is smooth and the spore mass is red. The presence of flagella, sporangia or sclerotia was not observed. Cell walls contained LL-diaminopimelic acid and ribose. The physiological properties and utilization of carbon sources of this strain are shown in Table 17 and 18, respectively.

Table 16. Cultural Characteristics of Strain S-35

Medium	Growth and reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	poor, pale pink	moderate, pinkish white	none
Glucose-asparagine agar	poor, lemon yellow	none	pale lemone
Glycerol-asparagine agar	poor, pale lemone	none	none
Starch-yeast extract agar	good, brownish yellow	abundant, reddish gray	none
Tyrosine agar	good, brown	scant, white	none
Nutrient agar	moderate, pale ivory	none	none
Yeast extract-malt extract agar	good, yellowish brown	abundant, reddish gray	brownish yellow
Oat meal agar	good, yellowish ivory	abundant, reddish gray	none

Table 17. Physiological characteristics of Strain S-35

Temperature range	13 - 33 C
Optimum temperature	25 - 30 C
Gelatin liquefaction	positive
Starch hydrolysis	positive
Milk peptonization	positive
Milk coagulation	positive
Melanoid pigment production	positive
O2 requirement	aerobic
H2S production	positive
NaCl tolerance	3%

Table 18. Sugar Utilization by Strain S-35

Sugars	Response
D-glucose	+
D-fructose	-
L-rhamnose	-
D-mannitol	-
L-arabinose	-
D-xylose	-
raffinose	-
sucrose	-
cellurose	-

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These properties suggest that strain S35 belongs to the genus *Streptomyces*. Among the known species of *Streptomyces* described in Bergey's Manual of Determinative Bacteriology,[187] the ISP reports[188] and other references,[189-193] strain S35 resembles *Streptomyces lavendulae* (Table 19, 20). Therefore, strain S35 is identified as a strain of *S. lavendulae*.

Table 19. Cultural Characteristics of Strain S-35, Str. lavendulae IFO 3177 and IFO 12789

Medium	Growth and Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate			
S-35	poor, pale pink	moderate, pinkish white	none
IFO 3177	poor, pale pink	moderate, pinkish white	none
IFO 12789	poor, pale pink	moderate, pinkish white	none
Glucose-asparagine			
S-35	poor, lemone yellow	none	pale lemone
IFO 3177	moderate, lemone yellow	poor, white	pale lemone
IFO 12789	moderate, lemone yellow	poor, white	pale lemone
Glycerol-asparagine			
S-35	poor, pale lemone	none	none
IFO 3177	poor, pale lemone	poor, pale lemone	none
IFO 12789	moderate, pale brown	poor, ivory	none
Starch-yeast extract			
S-35	good, brownish yellow	abundant, reddish gray	none
IFO 3177	moderate, brown	moderate, yellow	none
IFO 12789	good, green	abundant, yellowish gray	none
Nutrient			
S-35	moderate, pale ivory	none	yellow
IFO 3177	poor, pale ivory	none	pale brown
IFO 12789	moderate, brown	scant, gray	pale brown
Yeast-malt			
S-35	good, brownish yellow	abundant, reddish gray	brownish yellow
IFO 3177	good, brown	scant, ivory	brownish yellow
IFO 12789	good, dark brown	abundant, reddish gray	brownish yellow
Oat meal			
S-35	good, yellowish ivory	abundant, reddish gray	none
IFO 3177	moderate, yellow	moderate, lemone	none
IFO 12789	good, pinkish white	abundant, reddish gray	none

Table 20. Identification of strain S-35

	S-35	St. lavendulae
Physiological characteristics		
Optimum temperature		
25 °C	+	+
30 °C	+	+
37 °C	-	+
Gelatin liquefaction	positive	positive
Starch hydrolysis	positive	positive
Milk peptonization	positive	positive
Milk coagulation	positive	positive
Melanoid pigment production	positive	positive
H_2S production	positive	positive
Reduction of nitrate	positive	positive
Sugar utilization		
D-glucose	+	+
D-fructose	-	-
L-rhamnose	-	-
D-mannitol	-	-
L-arabinose	-	-
D-xylose	-	-
inositol	-	-
raffinose	-	-
sucrose	-	-
cellulose	-	-

Structure of S35-C2

The physicochemical properties of S35-C2 are as follows: mp 151-153 °C; UV λ_{max} (EtOH) nm: 223 ; IR $\tilde{\nu}_{max}$ (KBr) cm^{-1}: 3360, 3179, 1640, 1497, 1417, 1290, 1184, 1134, 1072. The molecular formula was determined by MS [m/z: 135 (M$^+$)] and elementary analysis (Found: C, 70.58; H, 6.77; N, 10.43. Calcd. for C_8H_9NO: C, 71.09; H, 6.71; N, 10.36 %). S35-C2 was positive to 2,4-DNPH (yellow), p-DABA (brown) and ninhydrin (purple red), which was indicated the presence of an amide group (IR 3360, 3179 and 1640 cm^{-1}) . 1H-NMR (CD3OD): 3.49 (2H, s), 7.28 (5H, m); 13C-NMR (CD3OD): 44.2 (t), 128.7 (d), 130.3 (d), 130.9 (d), 137.7 (s), 177.7 (s). On the basis of these data, S35-C2 were identified as phenylacetamide (Fig. 41). Phenylacetamide was also isolated from *Actinomyces* N-29 in 1963[194)] and *Streptomyces alboniger* in 1973,[195)] respectively. Furthermore, phenylacetamide is known as a plant growth regulator.[194,196)]

$$CH_2CONH_2$$
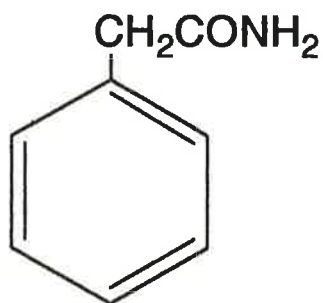
Phenylacetamide (S35-C2)

Fig. 41 The Structure of S35-C2

Effect of S35-C2 on sterility in rice

S35-C2 exhibited the rice sterility of 10.2 % and 53.5 % at the concentrations of 1000 and 3000 mg/l, respectively. Furthermore, the number of seeds, the spike length, the culm length and the weight of ripe seeds of rice plants treated with 3000 mg/l of S35-C2 were the same as those of control (Table 21).

Table 21. Mean Number of Seeds, Sterility, Spike Length, Culm Length and Weight of Ripe Seeds of "Yamabiko" Rice as Affected by S35-C2 at Meiosis

| Chemical		Number of seeds (no./spike)	Sterility (%)	Spike length (cm)	Culm length (cm)	Weight of ripe seeds (g, 1000^{-1})
S35-C2	1000 ppm	65	10.2 a[a]	17.5	55.4	23.9
	3000	67	53.5 b	17.3	60.9	23.2
Control		68	4.4 a	16.8	55.5	25.3

[a] Means in a column followed by the same letter are not significantly different at the 5 % level of significance by Duncan's Multiple Range Test.

*Physiological and biochemical characteristics of sterility induced by S35-C2*

GS activity, ammonia content and ATPase activity in panicles treated with S35-C2 at a concentration of 3000 mg/l were as the same level as those of control (Fig. 42).
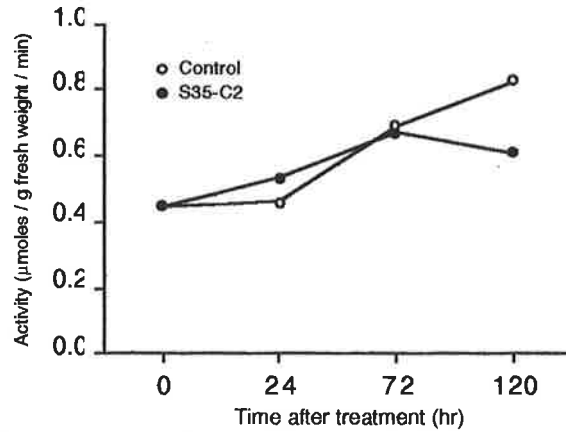
Time Course of Glutamine Synthetase in Panicles
of Rice Plants Treated with S35-C2
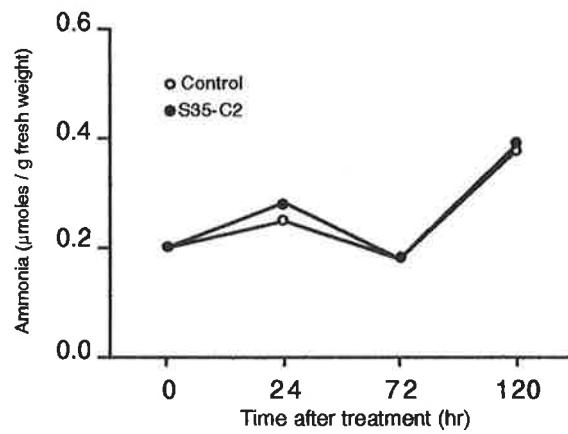
Time Course of Ammonia Content in Panicles
of Rice Plants Treated with S35-C2
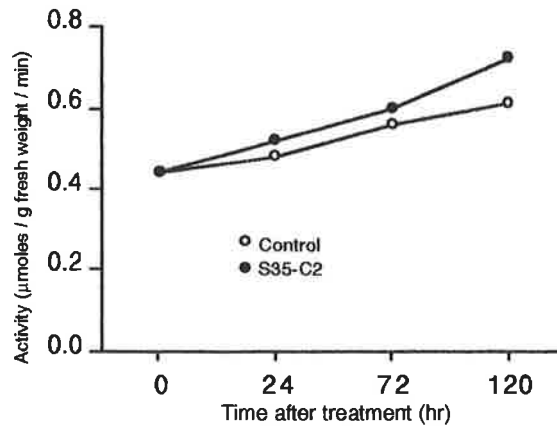
Time Course of ATPase Activity in Panicles
of Rice Plants Treated with S35-C2

Fig. 42 Effects of S35-C2 on GS Activity, Ammonia Content,
and ATPase Activity in Panicles of Rice Plants

9 2

The decrease of protein nitrogen in panicles was caused by treatment with 3000 mg/l of S35-C2 (Fig. 43). However, the protein nitrogen in leaves and culms treated with S35-C2 was the same as that of control (Fig. 43). On the other hand, the increase of free amino acids content in panicles was shown by treatment with 3000 mg/l of S35-C2 (Fig. 43). These results suggest that protein synthesis in panicles is suppressed by S35-C2 treatment.

Figure 44 show the component of various carbohydrates such as the reducing sugar, non-reducing sugar, starch and hemicellulose in panicles treated with 3000 mg/l of S35-C2 and an untreated panicles. The non-reducing sugar in panicles treated with 3000 mg/l of S35-C2 increased about 1.5 times as much as that of control. Furthermore, the non-reducing sugar in leaves and culms treated with 3000 mg/l of S35-C2 increased about 2 times as much as that of control (Fig. 44).

According to the paper chromatography, it was estimated that the non-reducing sugar in tissues of S35-C2 treatment was only sucrose and the reducing sugars were glucose and fructose. These results suggest that the accumulation of the non-reducing sugar by treatment with S35-C2 affected the carbohydrate matebolism of panicle, leaf and culm of the rice plant.

The decreases of chlorophyll in leaves treated with bialaphos at the concentrations of 1000 and 3000 mg/l were as the same tendency as that of control (Fig. 45).
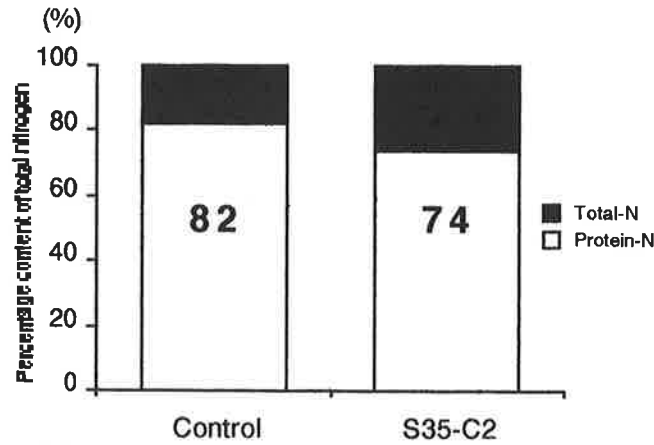
93

Effects of S35-C2 on Nitrogen Content in Panicles of Rice Plants
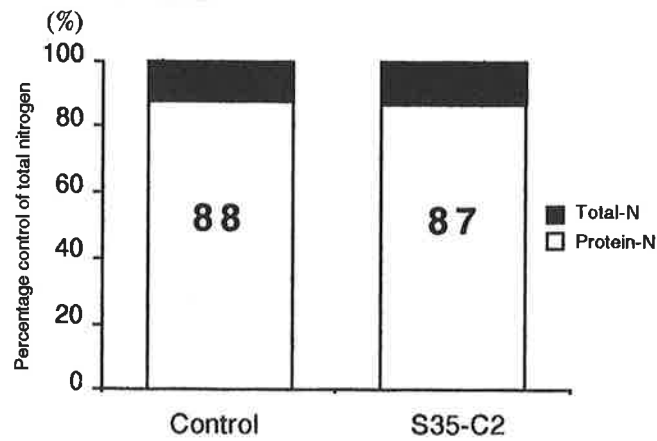
Effects of S35-C2 on Nitrogen Content in Leaves and Culms of Rice Plants
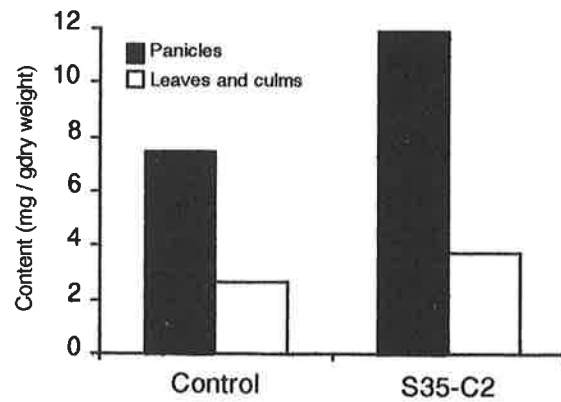
Effects of S35-C2 on Free Amino acids in Panicles and, Leaves and Culms of Rice Plants

Fig. 43 Effects of S35-C2 on Protein Nitrogen and Free Amino acid Content in Panicles, Leaves and Culms of Rice Plants

94

(%)

Percentage content of carbohydrates

Reducing sugar
Non-reducing sugar
Starch
Hemicellulose

Control   S35-C2

Effects of S35-C2 on Various Carbohydrates in Panicles
of Rice Plants

(%)

Percentage content of carbohydrates

Reducing sugar
Non-reducing sugar
Starch
Hemicellulose

Control   S35-C2
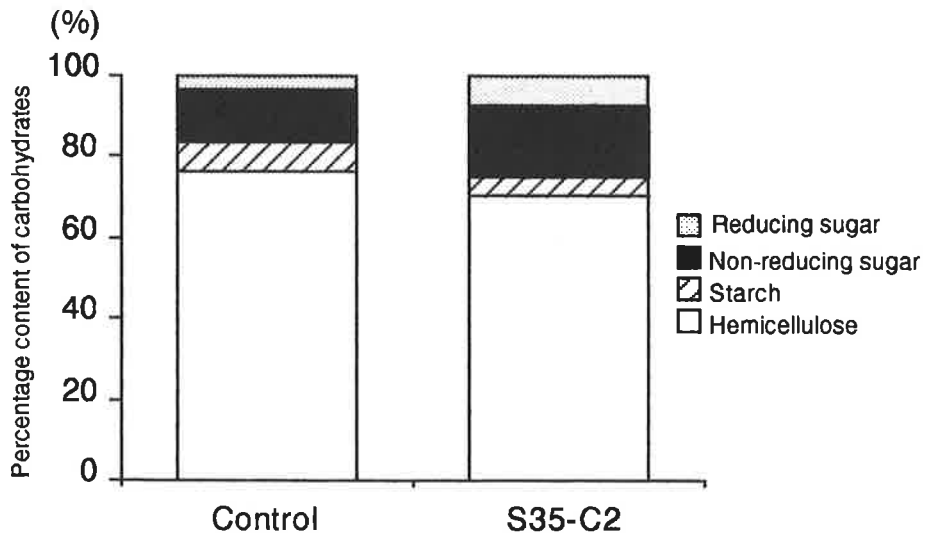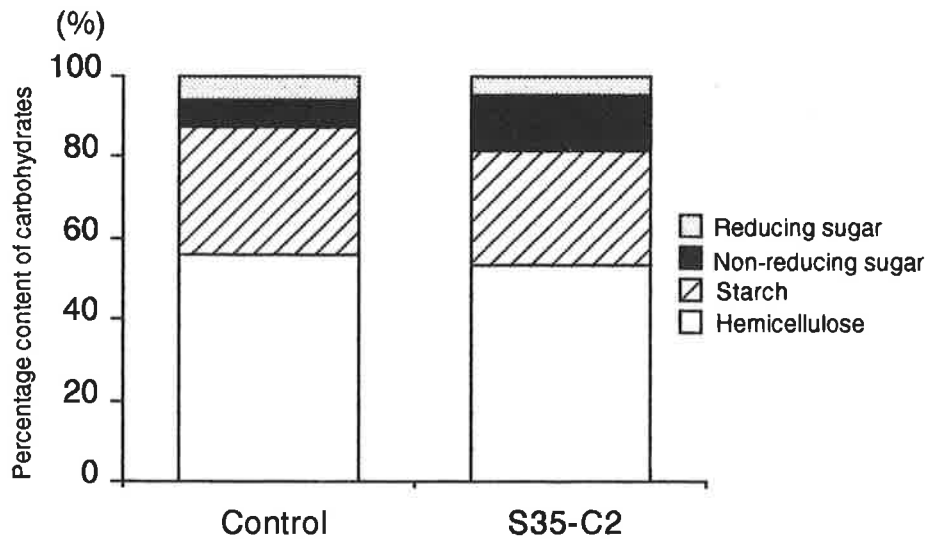
Effect of S35-C2 on Various Carbohydrates in Leaves and Culms
of Rice Plants

Fig. 44 Effects of S35-C2 on Various Carbohydrates
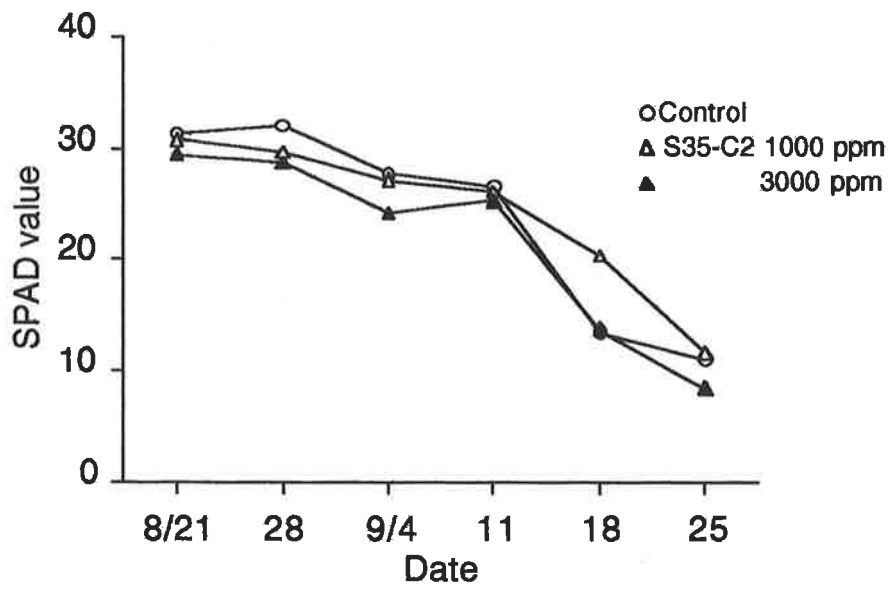in Panicles, Leaves and Culms of Rice Plants

Fig. 45 Effect of S35-C2 on Chlorophyll in Leaves of Rice Plants

Changes of free amino acids in anthers

The total contents of free amino acids in anthers with and without S35-C2 treatment were 17.20 nM and 26.10 nM per anther, respectively. The rate of proline was about a half of free amino acids in control anthers. Although the role of proline in plants were known to be utilized in new protein synthesis[164] and used as an energy source,[165] the role played by proline content was not clearly understood in connection with pollen fertility.[131,166]

The untreated anthers had about 1.4 times more proline than the anthers treated with S35-C2, while the treated anthers had the same aspartic acid and glutamic acid as the untreated anthers. On the other hand, the treated anthers had about 1.7 times more alanine than the untreated anthers (Table 22). In the free amino acids composition of leaves and culms, the increase of tyrosine and the decrease of alanine were shown in the leaves and culms treated with S35-C2 at a concentration of 3000 mg/l. The untreated leaves and culms had about 1.5 times more alanine than those treated with S35-C2, while the treated leaves and culms had about 1.9 times more tyrosine than the untreated leaves and culms. The treated leaves and culms had the same proline as the untreated leaves and culms (Table 23). From these results, the changes of free amino acids composition of anthers, leaves and culms treated with S35-C2 are different from those treated with bialaphos. Since GS inhibitory activity and the free amino acids composition of the rice plants treated with S35-C2 are different from those treated with bialaphos, the action mechanism of S35-C2 on rice sterility may be different from that of bilaphos.

Table 22. Free Amino acids Composition (%) of Anthers
Treated with S35-C2

Amino acids	Control	S35-C2 3000 mg / l
Aspartic acid	2.02	1.89
Threonine	5.63	5.97
Serine	6.28	5.74
Glutamic acid	2.51	2.33
Proline	51.04	37.33
Glycine	2.12	1.89
Alanine	18.21	29.98
Cysteine	0.24	0.34
Valine	2.77	3.94
Methionine	0.29	0.42
Isoleucine	0.45	0.81
Leucine	0.75	1.39
Tyrosine	1.18	1.22
Phenylalanine	0.82	1.57
Histidine	1.73	1.66
Lysine	1.89	1.43
Arginine	2.07	2.09

Table 23. Free Amino acids Composition (%) of Leaves and Culms Treated with S35-C2

Amino acids	Control	S35-C2 3000 mg / l
Aspartic acid	2.91	3.86
Threonine	4.46	6.04
Serine	5.28	3.93
Glutamic acid	6.31	6.09
Proline	13.00	13.04
Glycine	3.73	2.58
Alanine	28.39	18.61
Cysteine	1.40	tr.
Valine	7.71	9.42
Methionine	tr.	tr.
Isoleucine	1.23	2.21
Leucine	1.28	3.95
Tyrosine	12.36	22.75
Phenylalanine	2.26	1.98
Histidine	4.39	0.65
Lysine	1.12	4.89
Arginine	4.17	tr.

tr. Trace.

Effect of S35-C2 on the growth of rice seedlings

Figure shows the growth of rice seedlings treated with S35-C2 at the concentrations of 10 to 3000 mg/l (Fig. 46). The growth of rice seedlings treated with S35-C2 at the concentrations of 10 to 1000 mg/l were as the same level as that of control. However, the growth of rice seedlings treated with S35-C2 at a concentration of 3000 mg/l was inferior to that of control. In particular, S35-C2 exhibited the root growth inhibition of 91 % at a concentration of 3000 mg/l.

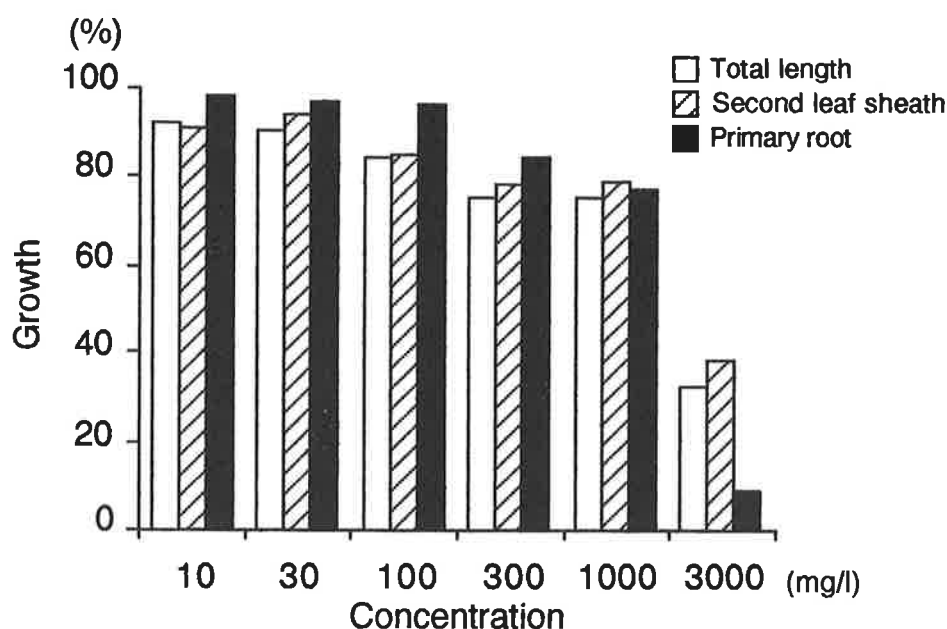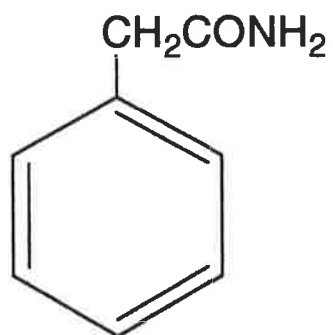However, it is not understood yet what is the mode of action of S35-C2 on the growth of rice seedlings.

Fig. 46 Effect of S35-C2 on the Growth of Rice Seedlings
(cv Yamabiko)

Conclusion

The utilization of heterosis for rice result in progeny with higher yield, increased resistance to disease, and enhanced performance in different environments compared with the parental lines. The chemical induction of male sterility in rice is potentially atractive, because the utilization of heterosis for rice is limited. The limiting factor in breeding is the prolonged process of developing male-sterile and restore lines for hybridization, and manual emasculation is labour intensive. These disadvantages could be overcome if chemicals were used to induce male sterility.

Kasugamycin, AOA and bialaphos were found as the effective rice sterilants (Fig. 47). S35-C2, a metabolite from *Streptomyces lavendulae*, was also found an effective rice sterilant (Fig. 47). Furthermore, the physiological and biochemical characteristics of rice sterility induced by chemical treatment were investigated in detail.

CH₂CONH₂

S35-C2 (Phenylacetamide)

NH_2-O-CH_2-COOH

AOA

$CH_3-\overset{\overset{O}{\parallel}}{\underset{\underset{OH}{|}}{P}}-CH_2-CH_2-\overset{\overset{NH_2}{|}}{CH}-CONH-\overset{\overset{CH_3}{|}}{CH}-CONH-\overset{\overset{CH_3}{|}}{CH}-COOH$
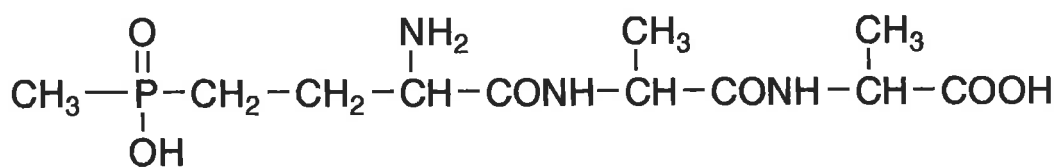
Bialaphos

Kasugamycin

Fig. 47 The Structures of S35-C2, Kasugamycin, AOA, and Bialaphos

1. Kasugamycin, AOA and bialaphos as the effective rice sterilants.

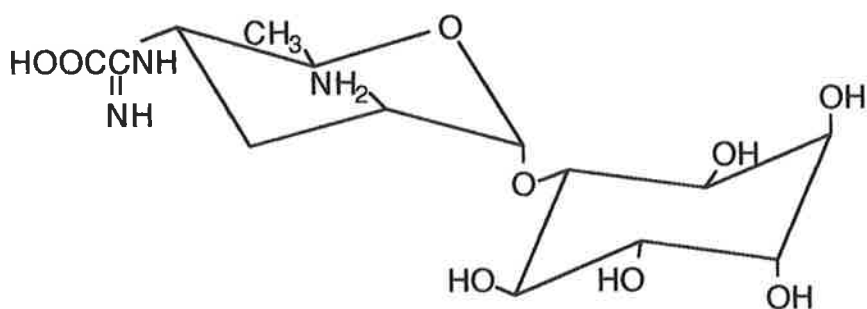Kasugamycin induced high level of rice sterility of 89.4 % at a concentration of 1000 mg/l when applied at meiosis. Rice plants treated with kasugamycin have no crop injury. In interactive effect between kasugamycin and plant hormones on rice sterility, the fertility was restored to rice only when NAA was applied simultaneously with kasugamycin. Kasugamycin, an inhibitor of protein synthesis, is a specific inducer of sterility in rice because erythromycin, kanamycin, tetracycline, and spectinomycin which are other inhibitors of protein synthesis have no effect on rice sterility.

AOA induced complete sterility in rice at a concentration of 1000 mg/l when applied at the panicle formation stage. However, rice plants treated with AOA have some crop injuries such as dwarf and straighthead. On the other hand, sex reversal from stamens to pistils in a glumous flower of rice plants was observed when AOA applied at a concentration of 1000 mg/l at the panicle formation stage. The number of pistils treated with AOA was 3.4 times as many as that of control. However, the conversion of sex expression was not observed at meiosis. Since AOA is an inhibitor of ACC synthesis, ACC or ethylene may play an important role in sex expression of rice at the initial reproductive stage.

Bialaphos is the most effective rice sterilant of the known chemicals used for inducing sterility, since bialaphos is almost complete sterility in rice at a concentration of 0.1 mM. Rice plants treated with bialaphos have not any crop injuries. Moreover, the results of the observations of glumous flowers treated with bialaphos suggest that the stamens are inhibited the normal growth but the pistils are not.

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Bialaphos, L-PTC and L-MSO are known to inhibit glutamine synthetase (GS). Although L-MSO produced no effect on sterility in rice, L-PTC induced the same level of sterility in rice as bialaphos. The results in vivo and in vitro of GS activities treated with bialaphos and L-PTC suggest that L-PTC is the real inhibitor of GS from rice plants and the real inducer of sterility in rice, since bialaphos is metabolized into L-PTC.

2. The physiological and biochemical characteristics of rice sterility induced by chemical treatment.

The endogeneous ammonia accumulation as a result of GS inhibition caused in panicles of rice plants treated with bialaphos and L-PTC treatment, respectively. Since sterility in rice is not caused by the exogeneous application of ammonia but caused by the endogeneous ammonia in panicles of rice plants, ammonia accumulation as a result of GS inhibition is a principal cause of inducing sterility in rice.

Although glutamine deficiency is caused as a result of GS inhibition, fertility in rice and GS activity in panicles of rice plants is restored by glutamine treatment simultaneously with bialaphos. This result suggest that GS inhibition treated with bialaphos is the reversible reaction.

The biochemical and physiological characteristics of rice sterility as a result of GS inhibition described below except ammonia accumulation.

The decrease of free amino acids content causes in the sterile anthers, and the most characteristic changes in the free amino acids in the sterile anthers are the decrease of proline and the increases of glutamic acid and aspartic acid. Furthermore, the result of iodine-

potassium iodide staining suggests that pollen sterility induced by bialaphos treatment is related to the absence or the decrease of pollen carbohydrate content.

The decrease of protein nitrogen and the increase of free amino acids content cause in the panicles, leaves and culms of the sterile rice plants. The increase of sucrose is observed in the panicles, leaves and culms of the sterile rice plants. Furthermore, the decrease of chlorophyll is observed in the leaves of the sterile rice plants.

Thus, the changes of amino acids composition and the decrease of protein are caused by the abnormal amino acid and carbohydrate metabolism in the sterile rice plants.

From several findings described above, figure 48 shows a possible mechanism of the biochemical alternations in the sterile anthers.
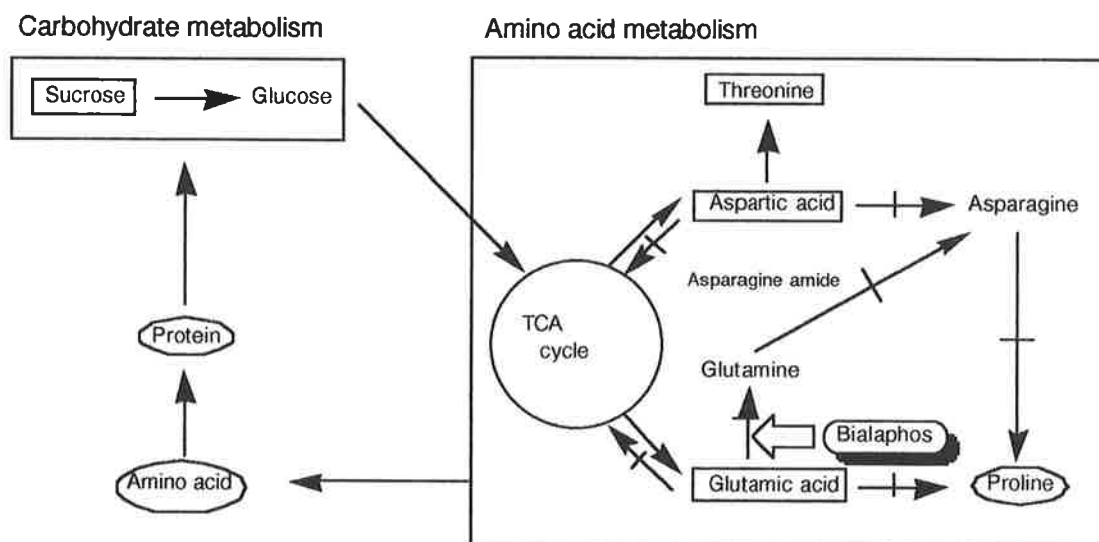

Fig. 48 The Possible Pathway of Abnormal Metabolism in the Sterile Anthers

□ : Accumulation, ⬭ : Decrease

3. S35-C2, an effective rice sterilant produced by *Streptomyces lavendulae*.

Since a high sterility in rice is induced by GS inhibitors such as bialaphos and L-PTC, screening for new GS inhibitor was tested by using culture filtrates from actinomycetes. Of about 600 actinomycetes tested, S35 was chosen because of a high GS inhibitory activity and a high yield of the extracts of this culture filtrate. For the identification of S35, the morphological characteristics, cell wall analysis, the physiological characteristics, and the utilization of carbon sources of strain S35 were examined. From the results in those analyses, strain S35 was identified as a strain of *Streptomyces lavendulae*. S35-C2 isolated from the culture filtrate of S35 was identified as phenylacetamide. Although S35-C2 does not exhibit GS inhibitory activity, S35-C2 exhibit the rice sterility of 53.5 % at a concentration of 3000 mg/l. Furthermore, rice plants treated with S35-C2 have not any crop injuries. The results of the physiological and biochemical characteristics of the sterile rice plants induced by S35-C2 show that the decrease of protein and the abnormal carbohydrate metabolism are caused in the sterile rice plants.

Kasugamycin, AOA, bialaphos, and S35-C2 will be provided with new structural classes which can serve as models for the syntheses of novel effective rice sterilants. The studies of the physiological and biochemical characteristics of rice sterility induced by chemical treatment will contribute to the elucidation of the reproductive functions in higher plants.

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