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TISSUE-SPECIFIC CHANGES OF GLUTAMINE SYNTHETASE ACTIVITY IN OATS AFTER RHIZOSPHERE INFESTATION BY *Pseudomonas syringae* pv. *tabaci*.

Thmoas J. Knight [a1], Stephen Temple[2], Champa Sengupta-Gopalan[2], Scott N. Twary[3] and Pat Unkefer[3]

[1] Department of Biological Sciences, University of Southern Maine, Portland, Me 04103-2506

[2] Plant Genetic Engineering Laboratory, New Mexico State University, Las Curces, NM 88003

[3] CST 18, Los Alamos National Laboratory, Los Alamos, NM 87545

[a] To whom correspondance should be addressed.

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Pat Unkefer

ABSTRACT

Oats (*Avena sativa* L. *lodi*) tolerant of rhizosphere infestation by *Pseudomonas syringae* pv. *tabaci* when challenged by the pathogen experience tissue-specific alterations of ammonia assimilatory capabilities. Altered ammonia assimilatory potentials between root and leaf tissue result from selective inactivation of glutamine synthetase (GS) by the toxin Tabtoxinine-B-lactam (TBL). Root GS is sensitive and leaf GSs are resistant to TBL inactivation. With prolonged challenge by the pathogen root GS activity decreases but leaf GS specific activity increase. Higher leaf GS activity is due to decreased rates of degradation rather than increased GS synthesis. Higher leaf GS activity and elevated levels of GS polypeptide appear to result from a limited interaction between GS and TBL leading to the accumulation of a less active but more stable GS holoenzyme.

Tolerant challenged oats besides surviving rhizosphere infestation, experience enhanced growth. A strong correlation exists between leaf GS activity and whole plant fresh weight, suggesting that tissue-specific changes in ammonia assimilatory capability provides the plant a more efficient mechanism for uptake and utilization of nitrogen.

INTRODUCTION

Pseudomonas sryringae pv. *tabaci*, a tobacco leaf pathogen, produces the phytotoxin Tabtoxinine-ß-Lactam (TßL), which has glutamine synthetase (GS, EC 6.3.2.1) as its physiological target (Sinden and Durbin, 1968; Turner, 1981). TBL inactivation of GS is active-site directed, ATP-dependent and irreversible (Langston-Unkefer et al., 1987). Although *Pseudomonas syringae* pv. *tabaci* has a strict host specificity for tobacco, TßL has been shown to inhibit GS from a variety of plant species (Turner, 1981; Franz et al. , 1982; Knight et al., 1986; Bush et al. , 1987). Rhizosphere infestation of oats *Avena sativa* L. *lodi* by *P. syringae* pv. *tabaci* was found to be lethal to the plant (Knight et al., 1986). Plant death occurs when the bacteria, within the rhizosphere, produces TßL which is subsequently taken up by the roots resulting in inactivation of both root and leaf GS isoforms. Irreversible inactivation of all plant GS leads to rapid toxic accumulations of ammonium, with symptoms being expressed as generalized chlorosis of leaf tissue followed by cell necrosis and eventually plant death (Turner and Debbage, 1982; Knight et al., 1986).

Oats tolerant of rhizosphere infestation by *P. syringae* pv. *tabaci* and TßL have been obtained, so we now possess two variant oat populations: one being sensitive to rhizosphere infestation and TßL, the other being tolerant of pathogen and TßL (Knight et al., 1988). Plants of the tolerant population when challenged by *P. syringae* pv. *tabaci* for a prolonged period experience numerous biochemical and morphological changes. The key biochemical change in tolerant challenged oats (TC) is a tissue-specific alteration of ammonia assimilatory potentials between root and leaf tissues. Altered ammonium assimilatory capabilities in TC oats are a result of root GS remaining sensitive to inactivation by TßL , while leaf GSs are resistant to TßL inactivation. Consequently, tolerant oats challenged by the pathogen have greatly reduced root GS activities, but leaf GS specific activities increase (Knight et al., 1988).

Tolerant oats apparently survive rhizosphere infestation due to continued ammonia assimilation via the leaf GS/GOGAT cycle. However, resistance to TßL inactivation by these novel leaf GS isoforms and the reason for increased leaf GS specific activity in TC oats with prolonged exposure to *P. syringae* pv. *tabaci* are not well understood. We have focused on the possibility that increased leaf GS activity is

due to a unique limited interaction between TßL and the novel GS. Lack of any evidence to suggest that TßL activates existing GS enzyme, makes the most likely explanation for increased leaf GS activity a result of a TBL-mediated modification of protein turnover by either increasing GS synthesis or decreasing GS degradation.

Recent studies by others indicate that overall abundance of GS polypeptide in plant tissues is a function of concerted rates of GS synthesis and degradation, both of which are in turn effected by environmental and developmental factors. In *Pisium spp.* expression of the chloroplast GS gene leading to changes in GS polypeptide levels was found to be light-induced and possibly phytochrome regulated (Edwards and Coruzzi, 1989; Sakamoto et al., 1990). In *Phaleous spp,* changes in GS polypeptide levels and GS isoform profiles were correlated with GS mRNA abundance, suggesting that mRNA levels have a primary regulatory role in GS gene expression (Cock et al., 1991). However, in older tissues or during leaf senescence the correlation between GS mRNA levels and abundance of different GS isoform did not persist. It was suggested that translation efficiency, or altered GS degradation rates could also effect GS polypeptide levels in these older tissues (Cock et al., 1991). In wheat, developmental changes in abundance of leaf GS polypeptide have been shown to be, in part, a function of protelycic degradation of GS polypeptides by a wheat endopeptidase (Streit and Feller, 1983). Proteolytic inactivation of GS in leaf extracts could be delayed by stabilization of GS protein with addition of certain metabolites to the extract. Protective effects of these metabolites were found to result from interactions with GS protein rather than with the inactivating endopeptidase (Frohlich et al., 1994). Based on these observations altered rates of GS synthesis or GS degradation or a combination of the two could be responsible for the increased specific activity of leaf GS in TC oats.

Morphological changes that occur when TC oats experience prolonged challenge by *P. syringae* pv. *tabaci* are best described as enhanced vegetative growth. Nitrogen nutrition and its relationship to plant growth has been the subject of previous studies (Ingestad and Lund, 1979; Salsac et al. 1987). It has been proposed that plant growth is determined by current amounts of nitrogen within the plant, with relative growth rates being a linear function of internal nitrogen concentration (Argen, 1988). The implication of this being the rate of nitrogen uptake is regulated by current nitrogen status of the plant. How this regulation is achieved is not fully

understood. In barley seedlings it was shown that nitrate uptake induction and negative feedback of nitrate influx are independent processes. It was concluded that nitrate influx could be subject to negative feedback by nitrogen flux through the GS/GOGAT pathway and/or by levels of reduced nitrogen derivatives (Siddiqi et al., 1990). Another recent study with maize roots showed that high intracellular concentrations of glutamine and/or asparigine suppressed net nitrogen uptake, while conditions that decreased root glutamine and/or asparigine levels stimulated net nitrogen uptake (Lee et al., 1992). Assuming the only consequence of rhizosphere infestation by the TßL-producing pseudomonad is selective inactivation of root GS, then increased growth by TC oats could occur if the above mentioned tissue-specific change in GS activities between root and leaf tissues provides a more efficient process for nitrogen uptake and utilization. The ramification being altered ammonium assimilatory potentials between root and leaf tissues resulting from selective inactivation of GS by TBL influences the rates of nitrogen uptake and ultimately impacts plant growth.

This paper reports that elevated leaf GS activity in tolerant challenged oats is due to decreased rates of GS polypeptide degradation possibly resulting from a limited GS-TßL interaction. We also characterize changes in some growth parameters resulting from altered ammonia assimilatory potentials between root and leaf tissue and show the existence of a strong linear correlation between leaf GS activity and plant fresh weight.

MATERIALS AND METHODS

Growth Conditions

Oat (Avena sativa L. Iodi) seeds from sensitive and tolerant populations were vernalized for 4 d at 4°C and then germinated in the dark for 3 d at 25°C. Germinating seeds were transferred to plastic cups containing sterile acid washed sand (3 seedling/cup) and grown under a 16L/8D photoperiod at 25°C. Plants were surface irrigated every other day with 40 ml of one-quarter strength modified Hoagland nutrient solution, with nitrogen being supplied as 10 mM KNO_3. Nutrient feeding supplemented subsurface irrigation with dH_2O. Transfer of seedlings to sand culture represented day 1 of growth in all experiments.

Pseudomonas syringae pv. *tabaci* was grown on a gyratory shaker (250 rpm) at 24°C in 1L of Wooley's minimal medium (Wooley et al., 1955). When cell cultures approached late log phase, zinc activation of toxin synthesis was initiated by the addition of 100 ml of 25 uM $ZnCl_2$ solution to cultures (Durbin and Uchytil, 1984). Bacterial cells were collected by centrifugation (6,000 x g for 15 m) and resuspended in dH_2O to a cell density of 8.8×10^9 cells/ml. Rhizosphere infestation of 7 d old oats was accomplished by addition of 20 ml of the bacterial cell suspension to the sand surface of half the sensitive and tolerant plants, plants receiving pathogen challenge are designated sensitive challenged (SC) and tolerant challenged (TC) oats. The remaining unchallenged oats were identified as sensitive unchallenged (SUC) and tolerant unchallenged (TUC) oats.

Assays

All extraction procedures were carried out at 4°C. Leaf and root tissue extracts were obtained by macerating tissues in a cold motar with a pestle and [illegible] extraction buffer (50 mM Imidazole-HCl, pH 7.5, and 10 mM $MgCl_2$), at a ratio of 3 ml buffer to 1 g tissue. The macerate was centrifuged at 15,000 x g for 15 min and the supernatant was used as the tissue crude extract for enzyme activity determinations and enzyme degradation experiments. Glutamine synthetase activity of tissue extracts was determined by the ADP-Transferase

reaction (Shaprio and Stadtman, 1970). Protein content of tissue crude extracts was measured by the method of Bradford (1979). Chlorophyll content was measured in 80% (v/v) acetone leaf extracts (Arnon, 1949). Total nitrogen was determined by Kjeldahl digestion (McKenzie and Wallace, 1954) and ammonium was measured using a Orion Model 95-12 ammonia selective electrode.

Electrophoresis and Immunoblotting

Ethanol precipitated proteins of tissue extracts were solubilized in sample buffer containing (2% w/v SDS, 25 mM Tris pH 6.8, 10% w/v sucrose, 25 mM Dithiothretiol, 1 mM EDTA, and 0.02% Bromophenol blue), and boiled for 5 min. The solubilized proteins were separated by an SDS-PAGE system using a 12% acrylamide gel according to Laemmli (1970).

After electrophoresis, separated proteins in the gel were electrophoretically transferred onto nitrocellulose (Towbin et al., 1979). GS on the nitrocellulose was visualized by using chicken anti-GS antiserum as the primary antibody and Alkaline phosphatase-conjugated goat anti-chicken antiserum as the second antibody. Color was developed using 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and 0.33 mg/ml p-nitroblue tetrazolium chloride.

Isolation of Poly(A) RNA and Northern Blot Analyses

Total RNA was isolated, using LiCl precipitation procedure described by De Vries et al. (1982). Poly (A) RNA was isolated by subjecting the total RNA to poly (U) sepharose chromatography (Murray et al., 1981). Poly (A) RNA was fractionated on 1% agarose/formaldehyde gels and transferred to nitrocellulose. Hybridization was carried out in 50% formaide at 42 °C using standard conditions (Sambrook et al., 1989). Probes were prepared by labeling a gel purified *EcoR*1 DNA fragment encoding maize GS_2 cDNA from MGS1 (Snusted et al., 1988) using the random priming procedure (Feinberg and Vogelstein, 1983). The filters were washed three times with 2x SSC, 0.1% SDS at 42 °C for 20 min followed by one wash with 2x SSC, 0.1% SDA at 65 °C for 20 min and exposed to x-ray film.

GS Degradation Determinations

Endopeptidase activity specific for GS or the ability of an exogenous hydrolyzase to degrade GS was determined as follows. Crude tissue extracts containing either 200 ug of total protein from SC and TC extracts or 450 ug of total protein from SUC and TUC extracts were incubated for 6 h at 25 °C or incubated for 6 h at 25 °C in the presence of 7 mg of beaded agarose conjugated with insoluble α-chymotrypsin (22 units of chymotrypsin activity). Unequal amounts of total protein between unchallenged and challenged oat extracts were used to compensate for differences between initial levels of GS polypeptide of the extracts. After incubation extracts were centrifuged at 12,000 x g for 10 min and the supernatant was ethanol precipitated at -70 °C overnight. The precipitated proteins were solubilized, separated by SDS-PAGE and GS polypeptide levels were determined by western blotting methods as described above.

Effects of methionine sulfoxamine (MSX) on the rate of GS degradation was determined as follows. The roots of oat plants from both sensitive and tolerant populations were placed in aerated nutrient solution containing 0.1 mM MSX for 24 hours. Plants not treated with MSX were placed in aerated nutrient solution for 24 hours. Root and leaf tissue extracts of these plants were treated to the GS degradation procedures stated above, using 200 ug total protein for MSX treated plants and 400 ug total protein for control plants. After incubation the precipitated proteins were solubilized, separated, and GS polypeptide levels were determined by western blotting methods as described above.

Experimental Replication

TBL production by *Pseudomonas syringae* pv *tabaci* is extremely variable and because the extent of GS inactivation depends on the presence of TBL, the extent of GS inactivation varies between experiments. As a result, all experiments, except for GS mRNA transcription experiment, were performed at least three times with internal replication. The results presented are from representative experiments.

Results

Changes in GS Activity upon rhizosphere infestation by *Pseudomonas syringae* pv *tabaci*.

Rhizosphere infestation of sensitive and tolerant oat seedlings with the TßL-producing *Pseudomonas syringae* pv. *tabaci* results in a rapid decline of root GS activity (Fig 1a, Table 1). Leaf GS, from the two variant oat populations, has a different response to rhizosphere infestation by *P. sryingae* pv *tabaci*. In sensitive challenged oat seedlings (SC) leaf GS is inactivated by TBL, and like the root, leaf GS activity declines rapidly (Fig 1B, Table1). Leaf GS in tolerant challenged (TC) oat seedlings which is resistant to TBL inactivation increases with prolong exposure to the pathogen/toxin (Fig 1b, Table 1). At 11 and 14 days after rhizosphere infestation, leaf GS specific activity of TC oats is 43 and 60% higher than tolerant unchallenged oat (TUC), respectively. Activities of leaf GS in unchallenged sensitive (SUC) and tolerant (TUC) oat seedlings are nearly always identical (Fig 1b, Table 1). Also, when a tox$^-$ strain of *P. syringae* pv. *tabaci* was used to infest the rhizosphere the activities of all GS isoforms in TC oats were not significantly different than those of SUC and TUC plants (Table 1). These findings indicate the selection process for tolerance or the presence of the pseudomonad within the rhizosphere are not responsible for increased GS activity in leaf tissue of TC plants.

Accumulation of glutamine synthetase polypeptides in *Pseudomanas syringae* pv. *tabaci* infested oats.

Relative levels of GS polypeptide in root and leaf tissue of SUC, SC, TUC and TC oats 11 days after infestation were determined by SDS-PAGE and Western blotting analysis, probed with a polyclonal antibody specific for GS polypeptide. Polypeptide levels for root and leaf GS isoforms in pathogen challenged SC and TC oats were found to be dramatically elevated when compared to GS polypeptide levels of unchallenged SUC and TUC oats (Fig 2). Accumulation of GS polypeptide in SC and TC tissues but not in SUC or TUC tissues indicate that this response is due to TBL. Although, GS polypeptide

levels increase in root and leaf tissues of both SC and TC oats, only the leaf GS in TC plants remains catalyticaly active. Ion-exchange chromatography of leaf GS from TC plants has revealed that both GS_1 and GS_2 isoforms retained activity (data not shown) and earlier *in vitro* inactivation studies have shown that both forms of leaf GS in TC plants are resistant to inactivation by TßL (Knight et al., 1988). A 43% increase in specific activity of leaf GS in TC oats over TUC oats is not in accord with the dramatic increase in levels of GS polypeptide indicating that not all the GS protein in TC oat leaves is fully active.

Accumulation of GS polypeptide does not result from increased transcription of GS genes.

To test for the possibility that increased GS polypeptide levels in leaves of TC oats was due to increased rates of transcription, the relative levels of GS_2 transcripts in SUC, TUC, and TC plants were examined (Fig 3). Poly(A)-RNA isolated from leaves of SUC, TUC, and TC oats, 11 and 14 days after infestation of TC plants were subjected to northern blot hybridization, using a GS_2 cDNA probe (Snusted et.al., 1988). No discernible differences in hybridization levels were observed between any oat leaf tissues. Infestation with *P. syringae* pv. *tabaci* did not alter levels of GS transcript this observation indicates that GS transcription rates are uncoupled from observed increases in GS polypeptides and higher leaf GS specific activity of TC oats.

Accumulation of GS polypeptide due to decreased rates of GS degradation.

Cell free extracts of root and leaf tissue from SUC, SC, TUC, and TC oats show differential rates of GS degradation by oat endopeptidases and different levels of susceptibility to proteolytic degradation by chymotrypsin. Electrophoresis and western blot analysis of root and leaf extracts from unchallenged oats SUC and TUC incubated at 25^0C for 6 h (endopepitase activity) or incubated with chymotrysin attached to beaded agarose at 25^0C for 6 h revealed little or no detectable GS polypeptide (Figs 4a and 4b). In contrast, root and leaf extracts from challenged oats SC and TC when treated as above retained relatively large amounts of GS polypeptide. Since twice as much total

protein from SUC and TUC extracts were used during the incubation period than that of SC and TC shows GS in SUC and TUC extracts was readily degraded by the oat endopeptidase system and also by chymotrypsin but GS from challenged SC and TC extracts was not significantly degraded by either treatment (Fig 4a and 4b). However, some minor degradation of GS in the SC and TC extracts was observed in the chymotrypsin digest evident by the appearance of a third immunoreactive band appearing between the GS_2 and GS_1 polypeptide (Fig 4b).

Cell free leaf extracts of MSX treated plants from sensitive or tolerant oat populations when compared to non-treated plants reveals that GS polypeptide of MSX-treated plants was also resistant to degradation by the endopeptidase and chymotrypsin (Fig 5). Unlike TBL, MSX completely inhibited all GS isoforms including the TBL-resistant leaf GS isoforms of TC oats. MSX and TBL are both active site directed irreversible inhibitors of GS, this would suggest a common mechanism may be involved in protecting GS polypeptide from degradation.

Characterization of specific growth parameters between the populations of variant oats.

The effects of rhizosphere infestation by *Pseudomonas syringae* pv *tabaci* on growth of oat plants from sensitive and tolerant populations were characterized by measuring the following growth characteristics:. whole plant fresh weight, whole plant dry weight, root and leaf protein levels, leaf chlorophyll content, root and leaf total nitrogen content, and GS activities Rhizosphere infestation of TC oats with *Pseudomonas syringae* pv *tabaci* leads to significant increases in both fresh weight and dry weight as compared to the weights of oats not challenged by *P. syringae* pv *tabaci* (Table1). Leaf GS activity, leaf protein content, leaf total nitrogen content and chlorophyll levels also increase in TC plants. However, root GS activity decreases, while root protein levels are not significantly altered (Table1). No significant changes for these parameters were observed between unchallenged plants of the sensitive or tolerant populations, or with tolerant oats challenged with a tox^- strain of the pathogen. It appears that observed morphological changes

in TC oats result from a TBL-mediated alteration of ammonia assimilatory potential between root and leaf tissue due to loss of root GS activity and increased leaf GS activity.

Correlation between leaf GS activity and plant fresh weight

A strong correlation was found to exist between whole plant fresh weight and leaf GS specific activity. Comparison of GS specific activity and fresh weight of SUC, SC, TUC and TC oats of 18 day old plants has a correlation coefficient of r= 0.975 (r^2= 0.951), while comparison of 23 day old plants has a correlation coefficient of r = 0.949 (r^2= 0.901) (Fig 6). Unchallenged oats from both sensitive and tolerant populations have similar fresh weights and leaf GS activities. Challenged sensitive oats (SC) are extremely stressed due to decreased root and leaf GS activities and this stress is evident by decreased fresh weights. Challenged tolerant oats (TC) with higher leaf GS activities have significantly higher fresh weights. This strong correlation between leaf GS activity and whole plant fresh weight could indicate that increased leaf GS activity has a significant positive impact on plant growth.

DISCUSSION

Oat plants selected for tolerance to rhizosphere infestation by the tobacco leaf pathogen *Pseudomonas syringae* pv *tabaci* experience numerous biochemical and morphological changes with prolonged exposure to pathogen and TßL. The key biochemical change in challenged tolerant oats (TC) is a tissue-specific alteration in ammonia assimilatory capabilities resulting from decreased root GS activity and a concurrent increase in leaf GS specific activity. Tissue-specific changes in activities of GS in TC plants are a consequence of differential sensitivity by GS isoforms to TBL, with root GS being sensitive and leaf GS isoforms being resistant to TBL inactivation. TBL as the causal agent for tissue-specific changes in GS activities in TC plants and that these changes are not a result of the selection process or due to an unknown rhizosphere interaction between TC oats and pathogen, is supported by tolerant oat plants not challenged with the toxin-producing pseudomonad or tolerant oats challenged with a tox$^-$ strain of *P. syringae* pv.*tabaci* are nearly identical in all respects to sensitive unchallenged oats.

Dramatic increases in root and leaf GS polypeptide levels occurs when oats from both populations are challenged with the TBL producing *P. syringae* pv. *tabaci* . GS protein of challenged oats is also less susceptible to proteolytic degradation by endogenous endopeptidases or by chymotrypsin. This is in stark contrast to plants from unchallenged populations which accumulate relatively little GS polypeptide and the GS protein present is degraded by the oat endopeptidases and chymotyrpsin. No difference in GS mRNA levels between SUC, TUC and TC oats supports decreased rates of GS degradation rather than increased rates of GS transcription as being responsible for increased GS polypeptide. Plants from both populations when treated with MSX, another active-site directed inhibitor of GS, accumulate GS polypeptide which is also less sensitive to protoleytic degradation. Similar accumulations of GS polypeptide between TBL and MSX treated oats suggest the existence of a common protective mechanism between the two active-site inhibitors. Although, accumulation of GS polypeptide in root and leaf tissue of both sensitive and tolerant plants occurred when treated with either TBL or

MSX, only leaf GS isoforms of TC plants retained catalytic activity. Since only, TC oats accumulate GS polypeptide and retain leaf GS activity indicates that a GS-TBL interaction is required for decreased rates of degradation. However, retention of activity by this novel GS suggest that a only a limited interaction between GS and TBL is required to protect the GS holoenzyme from rapid proteolytic degradation.

Degradation of GS in microbial systems has been fairly well studied but this is not the case for GS of higher plants. Extracts of senescing wheat leaves were shown to have decreased rates of GS degradation when certain solutes (magnesium ion and lysine) were added to the extract with the protective nature of these solutes resulting from interactions with the substrate GS rather than with the degrading endopeptidase (Frohlich et al., 1994). Also, ATP was shown to be effective at protecting wheat GS from proteolytic degradation by chymotrysin but ADP and AMP were not (Feller and Keist, 1986). The mechanism by which these agents protect GS from degradation is not well understood.

Proteolytic degradation of bacterial GS is initiated by covalent modification of an active site histidine residue (Stadtman, 1990). Glutamate and MSX binding by bacterial GS protects this histidine residue from modification and subsequent protoelytic degradation (Ranizio et al.,1969; Levine, 1981; Stadtman, 1990). Another potential initiation step for proteolytic degradation of GS is a conformational change, leading to dissociation of subunits. Binding of MSX to *E. coli* GS results in conformational changes that effects catalytic cooperativity, subunit interactions ((Sharke, 1980; Wedler, 1982) as well as stabliization against subunit dissociation (Maurizi and Ginsburg, 1982). Cooperativity and stabilization, as a result of MSX binding, are to be expected, since GS active sites are formed in the interface between two subunits (Almassay, 1986). TBL, because of its similar mechanism of GS inactivation, should also result in cooperativity and stabilization after binding. Assuming the existence of similar mechanisms for initiation of proteolytic degradation between microbial and plant GS, then active-site directed inhibitors like TBL and MSX should also interefere with the initial step of plant GS degradation, as a consequence accumulation of GS polypeptide should occur.

Leaf GS isoforms in TC oats are novel because they are resistant to complete TBL inactivation. Resistance to inactivation by TBL is still not completely understood. *In vitro* inactivation studies with TBL reveals that GS from TC leaf tissue, partially purified through ion-exchange chromatography, will lose 15 to 20% of its activity. After this initial loss of activity this GS becomes resistant to further inactivation by TBL (Knight et al., 1988). A 15 to 20 % decrease in activity could represent a limited TBL-GS interaction where TBL could inactivate 1 or 2 active sites. Consequently, this initial interaction could lead to a conformational change which protects remaining free active sites from further TBL inactivation. This proposed limited interaction between GS active-sites and inhibitor is apparently unique to TBl, because MSX completely inactivates this novel GS. The reason for different degrees of sensitivity between leaf GS of TC oats and the two active-site directed inhibitors must lie with structural differences between the two inhbitors. TBL, because of its B-lactam ring structure, could experiences steric hindrance after the proposed conformational change resulting from the initial TBL-GS interaction. At the same time this initial TBL-GS interaction is capable of preventing initiation of GS degradation. The reason that leaf GS from TC oats is capable of only a limited interaction with TBL is presently under invesitgation. However, observed increases for leaf GS specific activity in TC leaf tissue, with prolonged exposure to the pathogen /TBL, is due to dramatic accumulation of a less active but more stable GS protein.

Glutamine synthetase as a key enzyme in plant nitrogen metabolism is supported by challenge with the pathogen/TBL being lethal to oats from the sensitive population. In contrast, TC plants which lose root GS activity but have increased leaf GS specific activity not only survive rhizosphere infestation but they experience what can best described as enhanced vegetative growth. Increased growth parameters used to characterize enhanced growth of TC oats over TUC oats are: fresh weight, dry weight, chlorophyll content, leaf nitrogen content and leaf soluble protein levels. Tolerant oats not challenged by the pathogen/ TBL or challenged with a tox$^-$ strain of the pathogen remain nearly identical in all growth parameters to unchallenged oats from the sensitive population. Also, a strong linear correlation between increased leaf GS specific activity and increased fresh

weight of oats was found to exist. It is unlikely that increased specific activity of leaf GS alone would significantly impact plant growth. This suggest that GS function is involved in coordinating activities of other enzymes involved in nitrogen metabolism. Plant nitrogen metabolism, by its sequential nature, requires that catalytic activities of enzymes involved in nitrate reduction and assimilation be coordinated to prevent accumulation of the toxic intermediates nitrite or ammonia. Increases in growth parameters, especially total leaf nitrogen content and soluble leaf protein content, and the correlation between leaf GS activity and fresh weight indicates that GS, besides being a primary enzyme for ammonia assimilation, may have a regulatory role in coordination of nitrogen flux. Tissue-specific alteration of ammonia assimilatory capabilities that occur in TC oats must provide the plant a more efficient means for uptake and utilization of nitrogen resources. How selective inactivation of GS and tissue-specific changes in ammonia assimilatory potential effects overall nitrogen metabolism and how roots continue to meet their nitrogen requirements when they have greatly reduced GS activity are presently being investigated.

Literature Cited

Agren GI (1985) Theory for growth of plants derived from the nitrogen productivity concept. Physiol Plant 64: 17-28.

Almassy RJ, Janson CA, Hamilin R, Xuong N-H, Eisenberg D(1986). Novel subunit interaction in the structure of glutamine synthetase. Nature 323:304-309.

Arnon DI (1949) Copper enzymes in chloroplast. Polyphenoloxidase in *Beta vulgaris*. Plant Physiolo 24: 1-15.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Bush DR, Durbin RD, Langston-Unkefer PJ (1987) *In vivo* inactivation of glutamine synthetase by tabtoxinine-B-lactam in *Zea mays* suspension culture cells. Physiol Molec Plant Pathol 31: 227-235.

Cock MJ, Brock IW, Watson AT, Swarup R, Morby AP, Cullimore JV. (1991) Regulation of glutamine synthetase genes in leaves of Phaseolus vulgaris Plant Mol Biol 17: 761-771.

De Vries SC, Springer J, Wessels JGH (1982) Diversity of abundance of mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta 156: 129-135.

Durbin RD, Uchytil TF (1984). The role of intercellular fluid and bacterial isolate on the *in vivo* production of tabtoxin and tabtoxinine-B-lactam. Physiol Plant Pathol 24: 25-31.

Edwards JW, Coruzzi GM (1989) Photorespiration and light act in concert to regulate the expression of the nuclear gene for chloroplast glutamine synthetase. Plant Cell 1: 241-248.

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13.

Feller U, Keist M (1986) Senescence and nitrogen metabolism in annual plants. In H Lambers, JJ Neeteson, I Stulen, eds, Fundamental, Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants, Martinus Nijhoff Publishers, Dordrecht, 219-234.

Frantz TA, Peterson DM, Durbin RD (1982) Sources of ammonium in oat leaves treated with tabtoxin or methionine sulfoximine. Plant Physiol 69: 345-348.

Frohlich V, Fischer A, Ochs G, Wild A, Feller U (1994) Proteolytic inactivation of glutamine synthetase in extracts from wheat leaves: Effects of pH, inorganic ions and metabolites. Aust J Plant Physiol 21: 303-310.

Ingestad T, Lund A (1979) Nitrogen stress in birch seedlings: I. Growth techniques and Growth. Physiol Plant 45: 137-148.

Knight TJ, Durbin RD, Langston-Unkefer PJ (1986) Effect of Tabtoxinine-B-lactam on nitrogen metabolism in *Avena sativa* L. roots. Plant Physiol 82: 1045-1050.

Knight TJ, Bush DR, Langston-Unkefer PJ (1988) Oats tolerant of Pseudomonas syringae pv. tabaci contain tabtoxinine-B-lactam-insensitive leaf glutamine synthetase. Plant Physiol 88: 333-339.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Langston-Unkefer PJ, Robinson AC, Knight TJ, Durbin RD(1987) Inactivation of pea seed glutamine synthetase by the toxin tabtoxinine-B-lactam. J Biol Chem 262: 1608-1613.

Lee RB, Purves JV, Ratcliffe RG (1992) Nitrogen assimilation and control of ammonium an nitrate absorption by maize root. J Exp Bot, 43: 1385- 1392.

Levine RL (1983) Oxidative modification of glutamine synthetase: I. Inactivation is due to loss of one histidine residue. J. Biol Chem 258: 11823-11827.

Maurizi MR, Ginsburg A (1982) Active site ligand stabilization of quatenary structures of glutamine synthetase from *Escherichia coli.* J Biol Chem 257: 7246-7251.

McKinzie HA, Wallace HS (1954) The Kjeldahl determination of nitrogen: a critical study of digestion conditions- temperature, catalyst, and oxicdizing agent. Aust J Chem 7: 55-70.

Murray MG, Peters DL, Thompson WF (1981) Ancient repeated sequences in the pea and mung bean genomes and implications for genome evolution. J Mol Evol 17: 31-42.

Ronzio RA, Rowe WB, Meister A (1969) Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. Biochem 8: 1066-1075.

Sambrook J, Fritsch EF, Maniatis T (1989) MOlecular cloning- A laboratory manual. Cold Spring Habor Laboratory Press, New York.

Sakamoto A, Takeba G, Shibata D, Tanaka K (1990) Phytochrome-mediated activation of the gene for cytosolic glutamine synthetase (GS1) during imbibition of photosensitive lettuce seeds. Plant Mol Biol 15: 317-323.

Salsac L, Chailos S, Morot-Gaudry J-F, Lesaint C, Jolivet E(1987) Nitrate and ammonium nutrition in plants. Plant Physiol Biochem 23: 805-812.

Shapiro BM, Stadtman ER (1970) Gultamine synthetase (*E. coli*). Methods Enzymol 17A: 910-922.

Sharke A, Whitley EJ, Ginsburg A (1980) Conformational differences between unadenylylated and adenylylated glutamine synthetase from *Escherichia coli* on binding L-methionine sulfoximine. J Biol Chem255: 581-589.

Siddiqi MY, Glass ADM, Ruth TJ, Rufty TW (1990) Studies of the uptake of nitrate in barley. I. Kinectics of $^{13}NO_3^-$ influx. Plant Physiol 93:1426-1432.

Sinden SL, Durbin RD (1968) Glutamine synthetase inhibition: possible mode of action of wild-fire toxin from *Pseudomonas tabaci.* Nature 219: 379-380.

Snustad DP, Hunsperger JP, Chereskin BM, Messing J. (1988) Maize glutamine synthetase cDNA's: Isolation by direct genetic selection in *Eschericia coli.* Genetics 120: 1111-1124.

Stadtman ER (1990) Covalent modification reactions are marking steps in protein turnover. Biochem 29: 6323-6331.

Streit L, Feller U (1982) Inactivation of N-assimilating enzymes and proteolytic activities in wheat leaf extracts: Effect of pyridine nucleotides and of adenylates. Experientia 38: 1176-1180.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354.

Turner JG (1981) Tabtoxin, produced by *Pseudomonas tabaci,* decreases *Nicotiana tabacum* glutamine synthetase in vivo and causes accumulation of ammonia. Physiol Plant Pathol 19: 57-67.

Turner JG, Debbage J (1982) Tabtoxin-induced symptoms are associated with accumulation of ammonia formed during photorespiration. Physiol Plant Pathol 20: 223-233.

Wedler FC, Sugiyama Y, Fisher KE (1982) Catalytic cooperativity and subunit interactions in Escherichia coli glutamine synthetase: binding and kinetics with methionine sulfoximine and related inhibitors. Biochem 27: 2168-2177.

Wooley DW, Schaffner G, Braun AC (1955) Studies on the structure of the phytopathogenic toxin Pseudomonas tabaci. J Biol Chem 215: 485-493.

Figure Legends

Figure 1. Glutamine Synthetase activity following rhizosphere infestation with *P. syringae* pv. *tabaci*.
A. Effect of rhizosphere infestation with *P. syringae* pv. *tabaci.* on root glutamine synthetase activity.
B. Effect of rhizosphere infestation with *P. syringae* pv. *tabaci.* on leaf glutamine synthetase activity. Sensitive unchallenged oats (SUC □), Sensitive challenged oats (SC ◆), Tolerant unchallenged oats (TUC ●), and Tolerant challenged oats (TC ▲).

Figure 2. Glutamine synthetase polypeptide levels in oat tissues. Analysis of GS polypeptide composition from oat tissues following rhizosphere infestation with *P. syringae* pv. *tabaci.* Total soluble protein extracts (250 mg/lane) from root tissue (lanes1-4) and leaf tissue (lanes 5-8) of oat plants 11 days after infestation by *P. syringae* pv. *tabaci* were separated by SDS-PAGE, transferred to nitrocellulose and the filter was probed with polyclonal GS-specific antibody. Lane load order is as follows: Sensitive unchallenged oats (SUC-lanes 1 root and 5 leaf), Sensitive challenged oats (SC-lanes 2 root and 6 leaf), Tolerant unchallenged oats (TUC-lanes 3 root and 7 leaf), and Tolerant challenged oats (TC-lanes 4 root and 8 leaf).

Figure 3. Effect of *P. syringae* pv. *tabaci* infestation of oat plants on the level of GS transcripts. Poly (A)-RNA (3 mg/lane) isolated from leaves of plants 11 and 14 days after infestation was fractionated on agrarose-formaldehyde gels, transferred to nitrocellulose, and hybridized with a maize GS_2 cDNA probe. Lane load order is as follows: Sensitive unchallenged oat leaf (SUC lanes 1 [11d] and 2 [14d], Tolerant unchallenged leaf (TUC lanes 3 [11d] and 4 [14d]), and Tolerant challenged leaf (TC lanes 5 [11d] and 6 [14d]).

Figure 4. Effect of infestation by *P. syringae* pv. *tabaci* on the stability of oat root GS polypeptide to proteolytic degradation. Total soluble protein of root extracts from unchallenged oats SUC and TUC (475 mg/lane) and from challenged oats SC and TC (250 mg/lane) after incubation at 25 C for 6h (lanes 1-4) or incubated at 25 C for 6h in the presence of

agrarose-bound chymotrysin (lanes 5-8) were separated by SDS-PAGE, transferred to nitrocellulose and the filters were probed with polyclonal GS-specific antibody. Lane load order is as follows: Sensitive unchallenged oats (SUC-lanes 1 and 5), Sensitive challenged oats (SC-lanes 2 and 6), Tolerant unchallenged oats (TUC-lanes 3 and 7), and Tolerant challenged oats (TC-lanes 4 and 8). Unequal loading of total protein between unchallenged and challenged oat extracts was done to compensate for different initial levels of GS polypeptide between the extracts.

Figure 5. Effect of infestation by *P. syringae* pv. *tabaci* on the stability of oat leaf GS polypeptide to proteolytic degradation. Total soluble protein of leaf extracts from unchallenged oats SUC and TUC (475 mg/lane) and from challenged oats SC and TC (250 mg/lane) after incubation at 25 C for 6h (lanes 1-4) or incubated at 25 C for 6h in the presence of agrarose-bound chymotrysin (lanes 5-8) were separated by SDS-PAGE, transferred to nitrocellulose and the filters were probed with polyclonal GS-specific antibody. Lane load order is as follows: Sensitive unchallenged oats (SUC-lanes 1 and 5), Sensitive challenged oats (SC-lanes 2 and 6), Tolerant unchallenged oats (TUC-lanes 3 and 7), and Tolerant challenged oats (TC-lanes 4 and 8). Unequal loading of total protein between unchallenged and challenged oat extracts was done to compensate for different initial levels of GS polypeptide between the extracts.

Figure 6. Effect of methionine sulfoximine on the stability of oat leaf glutamine synthetase polypeptide to proteolytic degradation. Total soluble protein of leaf extracts from unchallenged oats SUC and TUC (475 mg/lane) and from challenged oats SC and TC (250 mg/lane) after incubation at 25 C for 6h (lanes 1-4) or incubated at 25 C for 6h in the presence of agrarose-bound chymotrysin (lanes 5-8) were separated by SDS-PAGE, transferred to nitrocellulose and the filters were probed with polyclonal GS-specific antibody. Lane load order is as follows: Sensitive unchallenged oats (SUC-lanes 1 and 5), Sensitive challenged oats (SC-lanes 2 and 6), Tolerant unchallenged oats (TUC-lanes 3 and 7), and Tolerant challenged oats (TC-lanes 4 and 8). Unequal loading of total protein between unchallenged and

challenged oat extracts was done to compensate for different initial levels of GS polypeptide between the extracts.

Figure 7. Correlation between leaf glutamine synthetase and plant fresh weight. Correlation between oat leaf glutamine synthetase activity and whole plant fresh weight at 18 d (closed symbols) and 24 d (open symbols) after rhizosphere infestation with *P. syringae* pv. *tabaci.* Sensitive unchallenged oats (SUC □), Sensitive challenged oats (SC ◆), Tolerant unchallenged oats (TUC ○), and Tolerant challenged oats (TC ▲).

TABLE 1: Changes in Selected Growth Parameters of Oats after Rhizosphere Infestation by *Pseudomonas syringae* pv.*tabaci* .

Parameter	SUC	SC tox$^+$	TUC	TC tox$^+$	TC tox$^-$
Leaf GS (μmol mg^{-1} m^{-1}) 18 days*	1.290 ± 0.13	0.636 ± 0.09 {44%}	1.418 ± 0.06	2.502 ± 0.28 {143%}	1.407 ± 0.09 {92%}
Root GS (μmol mg^{-1} m^{-1}) 18 days*	1.924 ± 0.08	0.533 ± 0 .06 {28%}	1.943 ± 0.09	0.505 ± 0.04 {26%}	1.875 ± 0.09 {96%}
Fresh Weight (g) 18 days*	1.110 ± 0.19	0.485 ± 0.09 {44%}	1.256 ± 0.16	2.215 ± 0.21 {176%}	1.378 ± 0.33 {110%}
Fresh Weight (g) 34 days*	3.398 ± 0.64	ND	4.094 ± 0.80	6.223 ± 0.90 {152%}	ND
Dry Weight (g) 34 days*	0.326 ± 0.05	ND	0.360 ± 0.04	0.641 ± 0.08 {178%}	ND
Chlorophyll (mg gfw^{-1}) 24 days*	1.026 ± 0.07	0.466 ± 0.03 {45%}	0.977 ±. 0.02	1.250 ± 0.11 {128%}	0.998 ±0.06 {102%}
Leaf Protein (mg gfw^{-1}) 18 days*	6.157 ± 0.21	5.585 ± 0.35 {91%}	6.026 ± 0.44	8.804 ±. 0.21 {146%}	6.228 ±.0.36 {103%}
Root Protein (mg gfw^{-1}) 18 days*	2.339 ± 0.21	1.907 ± 0.26 {81%}	2.274 ± 0.18	2.154 ± 0.17 {95%}	2.246 ± 0.21 {99%}
Total Nitrogen Leaf (mg gdw^{-1}) 34 days*	26.56 ±1.58	19.09 ± 1.13 {72%}	27.88 ± 2.18	43.25 ± 2.52 {155%}	ND
Total Nitrogen Root (mg gdw^{-1}) 34 days*	15.83 ± 1.52	12.48 ± 0.91 {79%}	16.29 ± 0.77	15.06 ± 0.41 {92%}	ND

ND = Not Determined, { } = % of unchallenged plant, * = days after rhizosphere infestation

Fig 1A

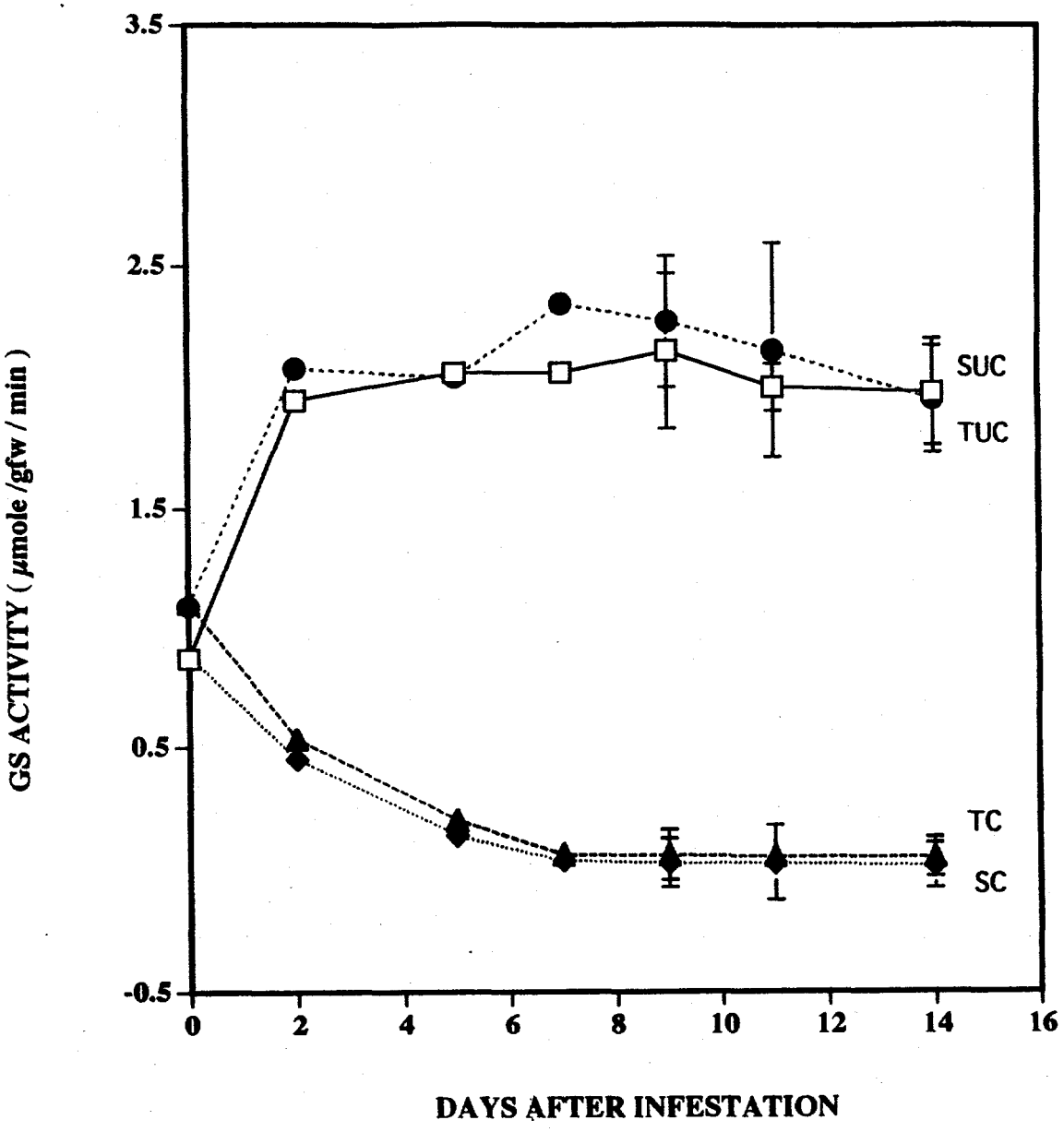

3.5
2.5
1.5
0.5
-0.5
0
2
4
6
8
10
12
14
16
SUC
TUC
TC
SC
GS ACTIVITY (μmole /gfw / min)
DAYS AFTER INFESTATION

Fig 1B

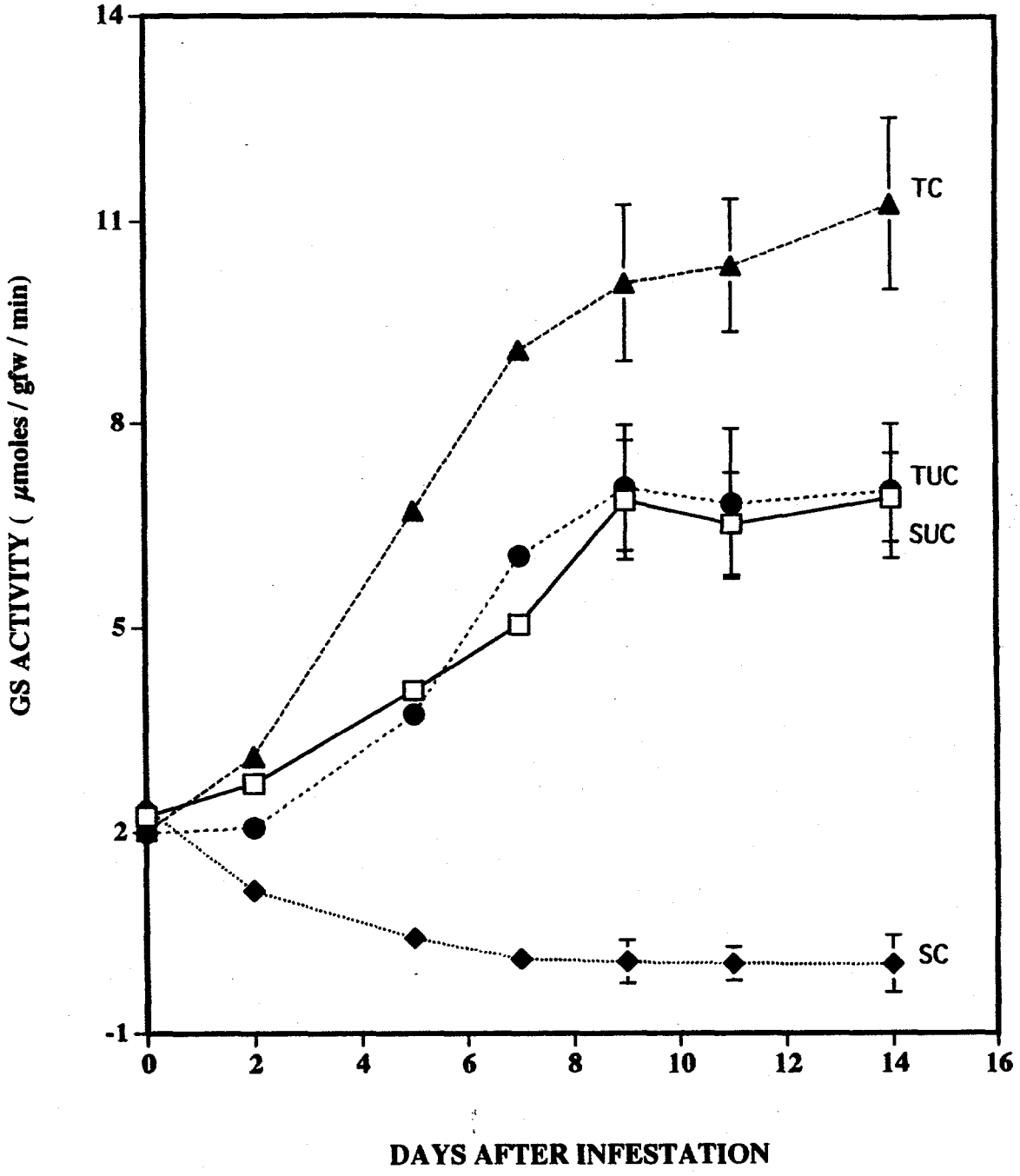
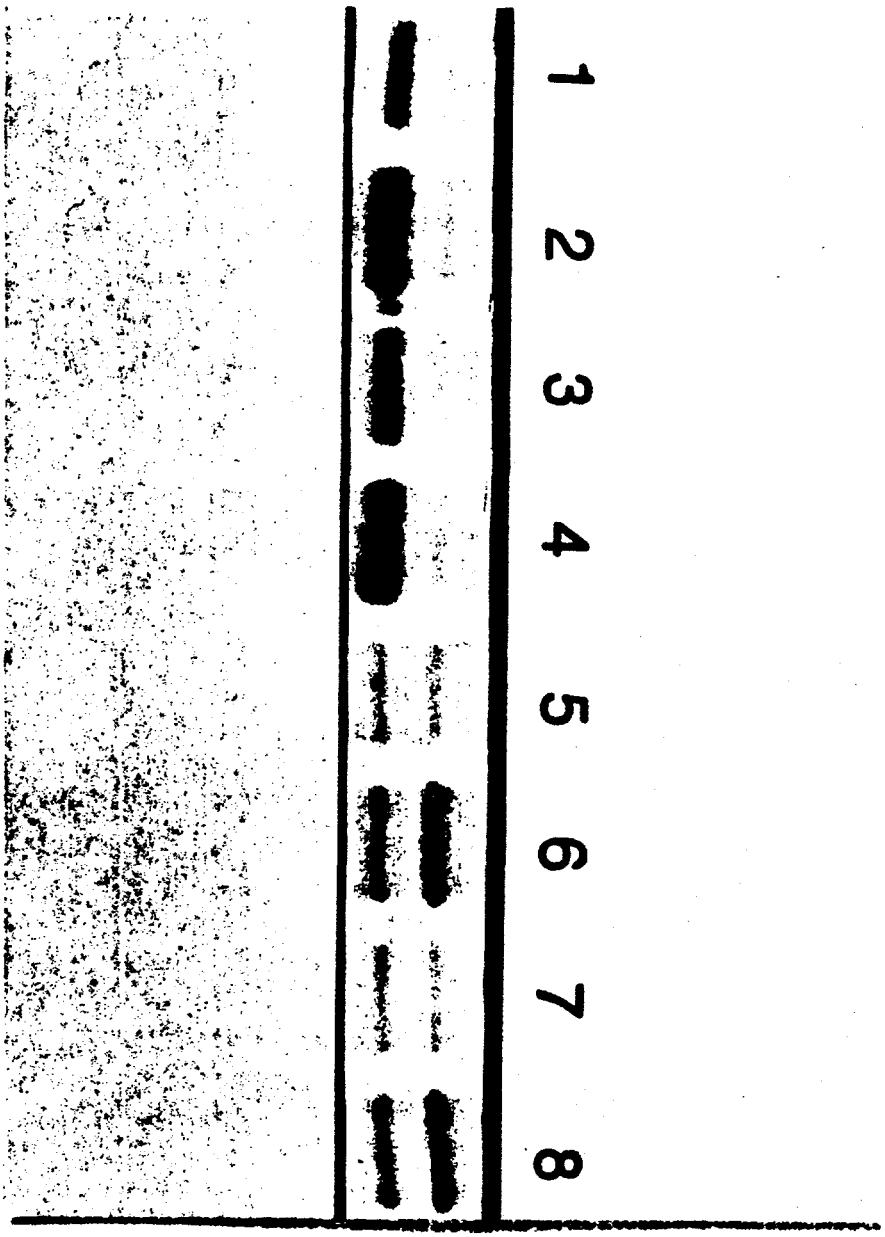

14
11
8
5
2
-1
0
2
4
6
8
10
12
14
16
TC
TUC
SUC
SC
GS ACTIVITY (μmoles / gfw / min)
DAYS AFTER INFESTATION

Fig 2



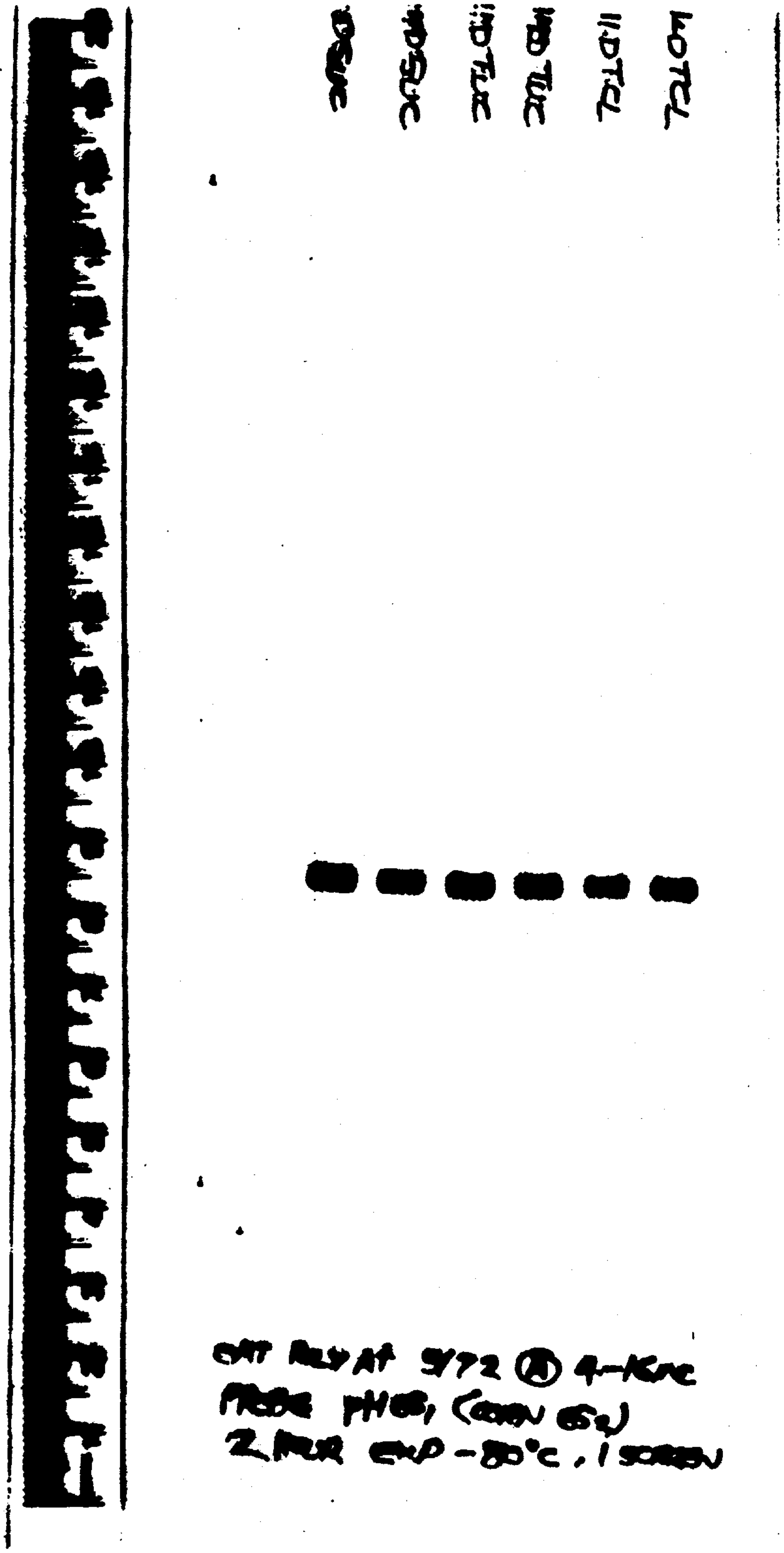


Fig 3

Fig 4

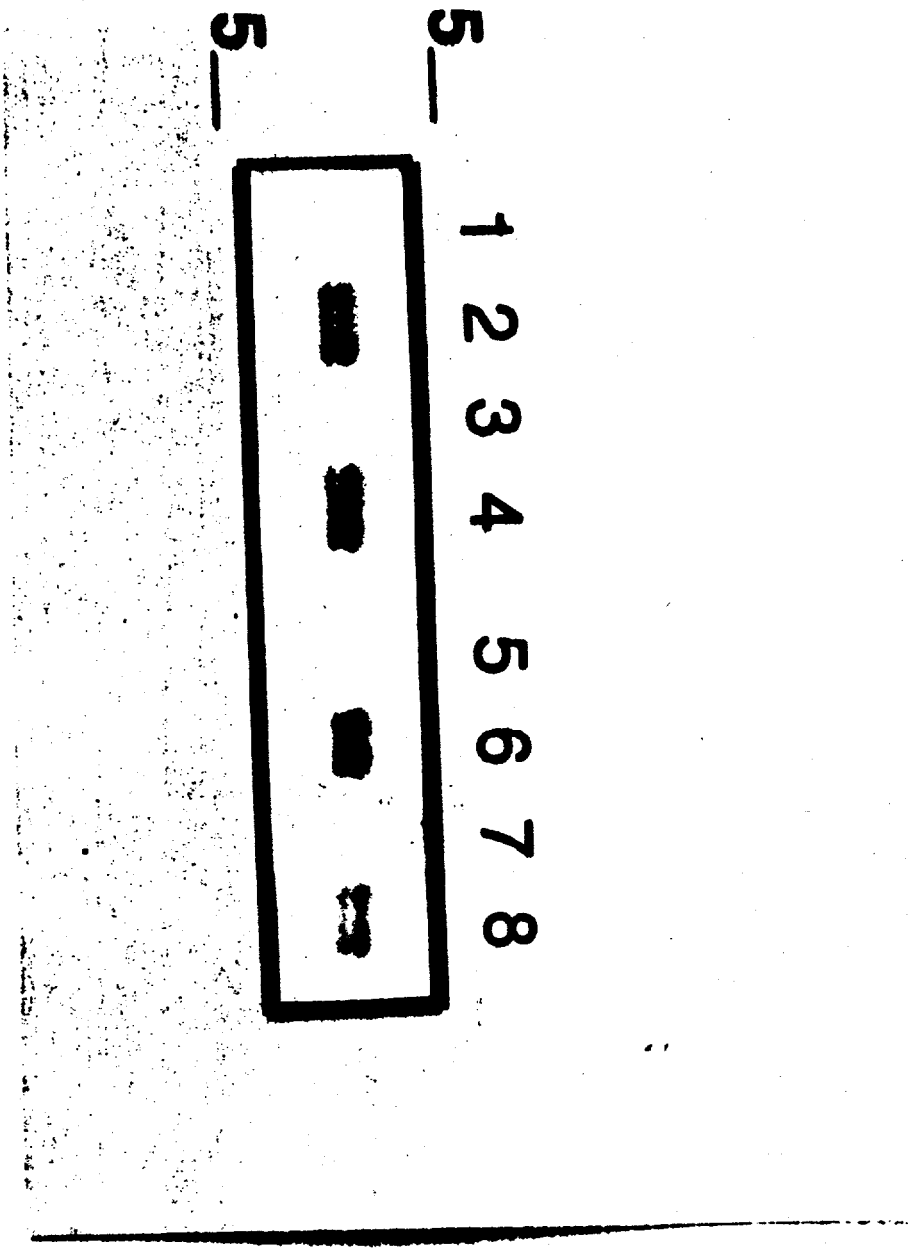
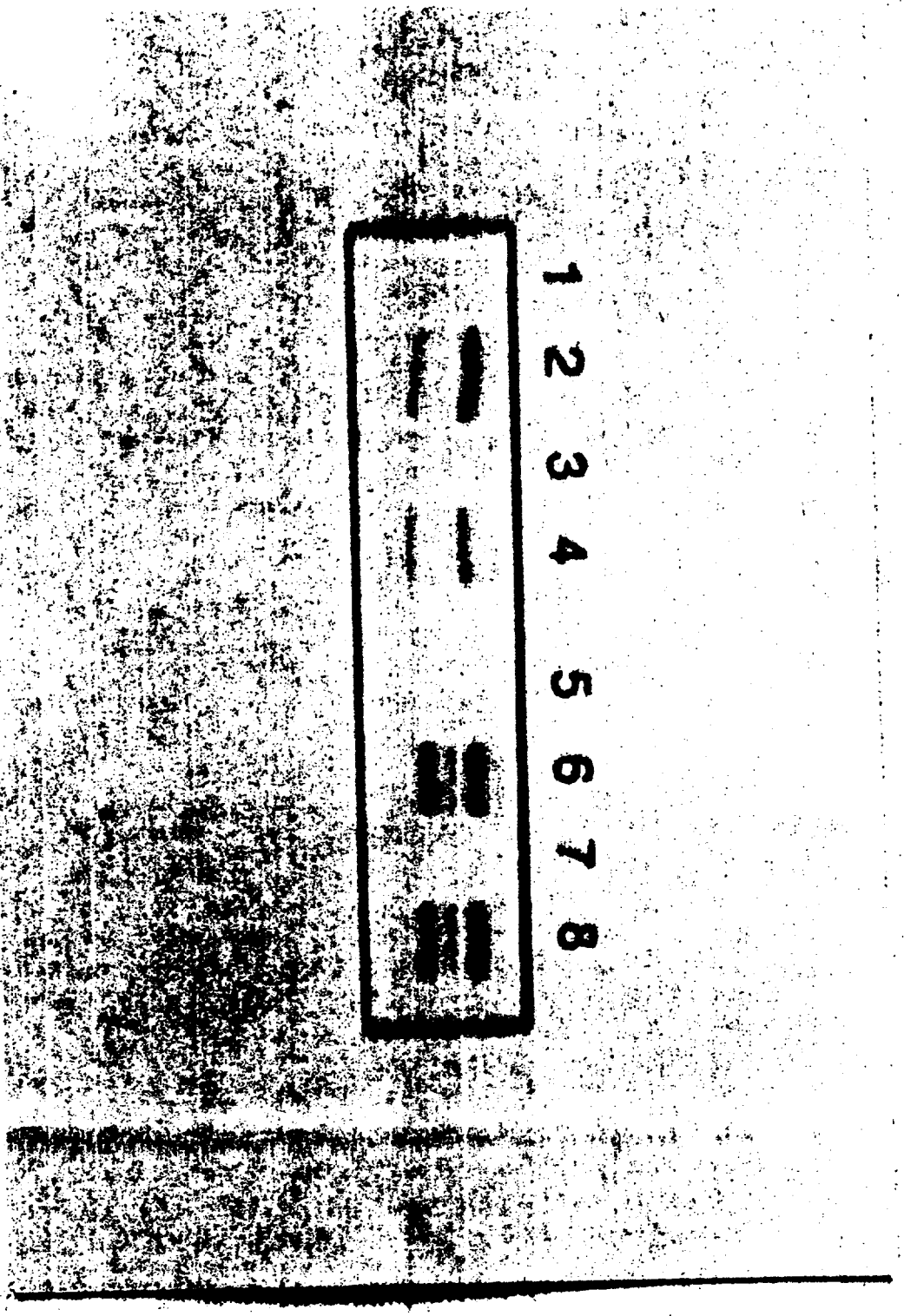


Fig 5



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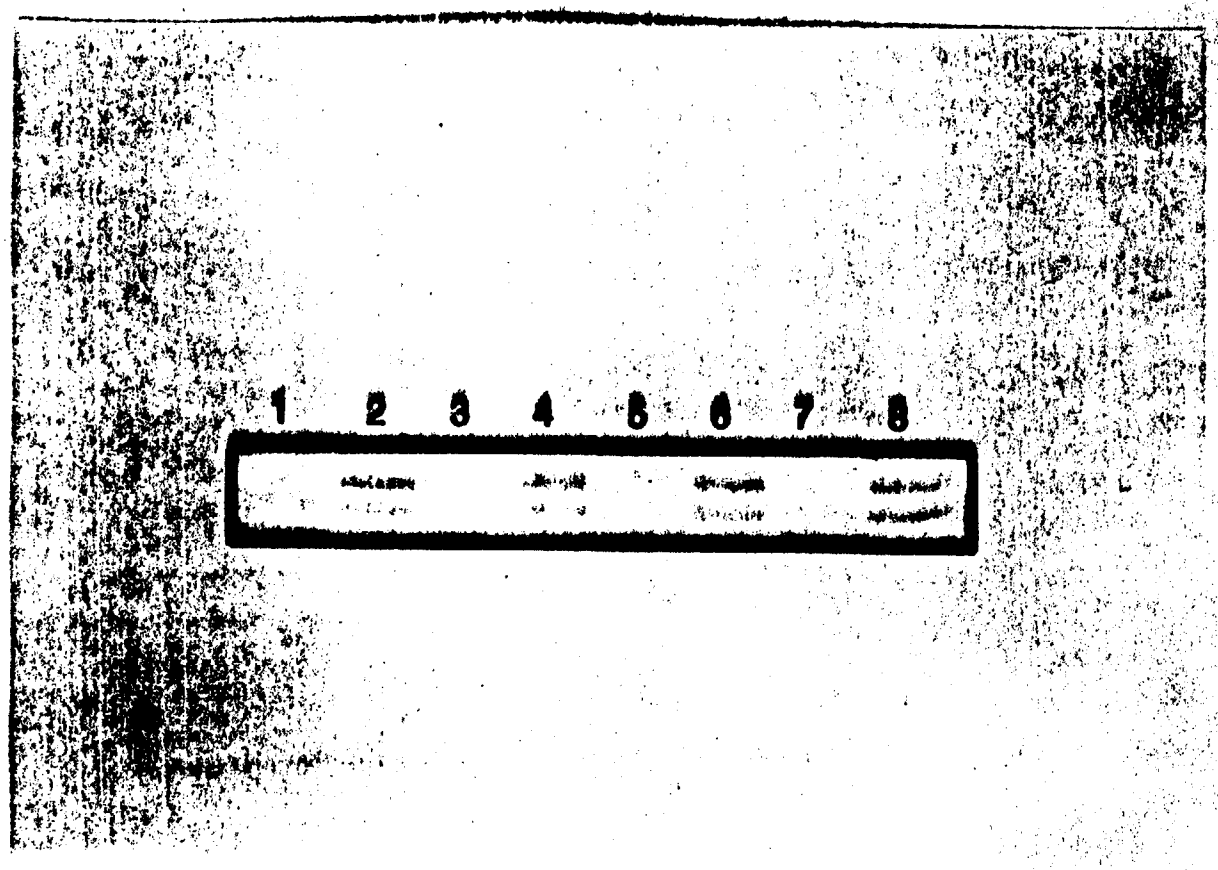
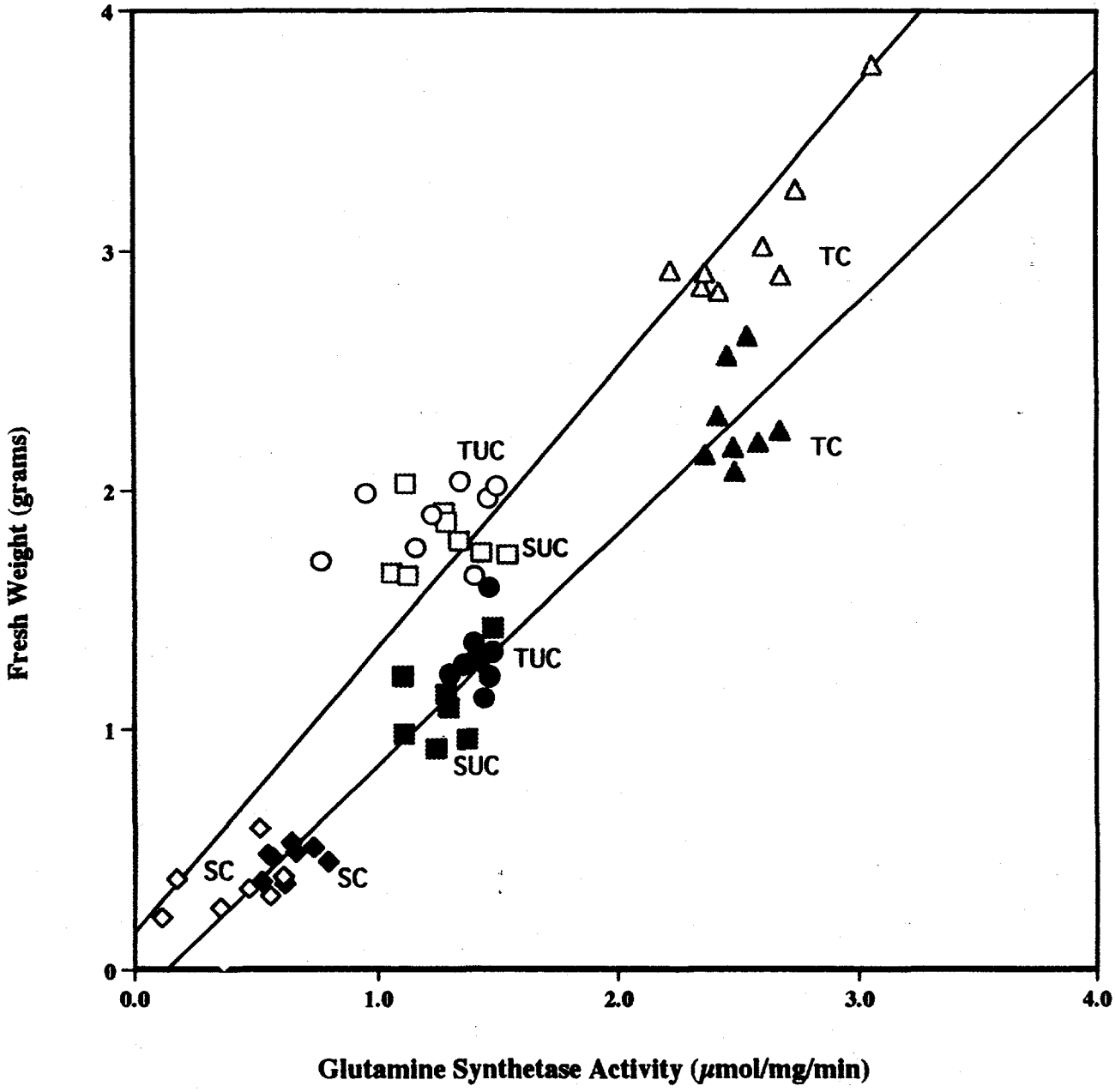


Fig 7


Fresh Weight (grams)
Glutamine Synthetase Activity (μmol/mg/min)
0
1
2
3
4
0.0
1.0
2.0
3.0
4.0
TC
TC
TUC
TUC
SUC
SUC
SC
SC

catalysts. Palladium catalysts have higher sulfur resistance than platinum. A Pd/TiO_2 catalyst was the most active and the least affected by sulfur among the three noble metal catalysts studied.

Mesoporous molecular sieves were shown to be active for the hydrocracking of bulky hydrocarbon molecules. Acidity is a key factor; it depends on the aluminum incorporation. Mesoporous molecular sieve catalysts also seem to be more stable—in terms of less coke formation—for the conversion of bulky hydrocarbons as compared to microporous molecular sieves.