Universidad Nacional Autónoma de México

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Comportamiento de las enzimas involucradas en la asimilación de amonio durante el estrés en Zea mays L. y Canavalia ensiformis

T E S I S

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INTRODUCCION.

Las plantas, debido a su imposibilidad de movimiento, tienen una reserva limitada de nitrógeno disponible para su desarrollo y crecimiento. Si a este hecho agregamos que se ven continuamente sometidas a condiciones desfavorables en su medio ambiente, entonces es de esperarse que aquellas plantas que resistan mejor estas condiciones y se adapten con mayor rapidez a los cambios puedan sobrevivir.

Aún cuando en la mayoría de las plantas la vía prioritaria de asimilación de amonio es el ciclo GS/GOGAT, se ha encontrado que la GDH puede estar funcionando bajo ciertas condiciones como son diferentes fuentes de nitrógeno, estrés de agua, etc. (Becana et al, 1984; Loyola-Vargas y Sánchez de Jiménez, 1984; Oaks y Hirel, 1985). También se ha observado en varios sistemas que diferentes tipos de estrés inducen cambios en el metabolismo de los azúcares, los cuales implican a su vez cambios de una vía metabólica a otra (Levitt, 1980).

Por otra parte, durante el estrés se acumulan diferentes compuestos nitrogenados como son aminoácidos, sales cuaternarias de amonio, alcaloides, etc., cuyas funciones aún están en controversia (Stewart y Larher, 1980). Es un hecho que el nitrógeno proveniente de procesos de tipo hidrolítico no llega a cubrir esta demanda, por lo que es necesario que haya una asimilación de novo de este elemento. La vía de asimilación de amonio que esté funcionando durante el estrés deberá tener características especiales y es probable que: a) la vía de asimilación sea diferente de la empleada en condiciones normales

ó b) que las enzimas sean de alguna forma distintas de aquéllas en plantas no estresadas.

En el presente trabajo, se estudiaron el comportamiento y algunas características de las enzimas involucradas en la asimilación de amonio en plantas de maíz y C. ensiformis sometidas, tanto a estrés de agua como de salinidad.

ANTECEDENTES.

1. Definición de estrés.

El estrés se define como cualquier cambio en las condiciones del medio ambiente que pudieran reducir o modificar de manera adversa el crecimiento, desarrollo o funcionamiento de la planta. Si este cambio es suficientemente severo, da lugar a un daño permanente o hasta la muerte.

2. Resistencia al estrés.

A la habilidad de una planta para sobrevivir en presencia de este cambio desfavorable y aún crecer, se le denomina resistencia al estrés. Por lo menos se han descrito dos tipos principales: la resistencia elástica, que es la medida de la habilidad de una planta para reducir o prevenir los cambios reversibles (químicos o físicos) cuando está expuesta a un estrés específico y la resistencia plástica, que es aquélla que presenta un organismo para reducir o prevenir cambios de tipo irreversible.

Debe tenerse en cuenta que aún cuando los cambios elásticos son reversibles, éstos pueden llegar a ser irreversibles (daño plástico) si la condición desfavorable está presente durante un tiempo prolongado.

Cuando un estrés específico actúa sobre una planta, puede dar origen a lesiones de diferentes maneras:

1. Puede producir un cambio directo más allá del límite

elástico de la planta y por tanto, un cambio plástico que induzca una lesión. Esta sería una lesión directa del estrés en cuestión, que puede reconocerse por la rapidez de su aparición.

2. Al inducirse un cambio elástico que es reversible, y en sí no dañino, pero que si se mantiene durante un tiempo largo puede dar lugar a un cambio plástico indirecto, el cual conducirá a la aparición de lesiones o en última instancia a la muerte de la planta. Esta es una lesión indirecta del estrés, que se caracteriza por la larga exposición al estrés antes de que se produzca la lesión, y
3. Cuando un estrés puede dañar a la planta, no por él mismo, sino al dar origen a un segundo estrés. A este daño se le denomina lesión secundaria del estrés.

3. Clasificación del estrés.

El estrés ambiental se ha dividido en dos grandes grupos: el estrés biótico y el estrés fisicoquímico. En la figura 1 se muestra como se han clasificado los diversos tipos de estrés que se han estudiado. Dentro del estrés fisicoquímico se encuentran 4 subgrupos principales y uno en el cual se han conjuntado a aquéllos que no entran en los otros subgrupos (Levitt, 1980).

En los tipos de estrés marcados con asterisco (*) se han encontrado dos mecanismos de resistencia: evitar el estrés o su tolerancia. En el primer caso, se evita llegar al equilibrio termodinámico con el estrés. La planta que posee este mecanismo es capaz de excluir al estrés, ya sea parcial o totalmente, por

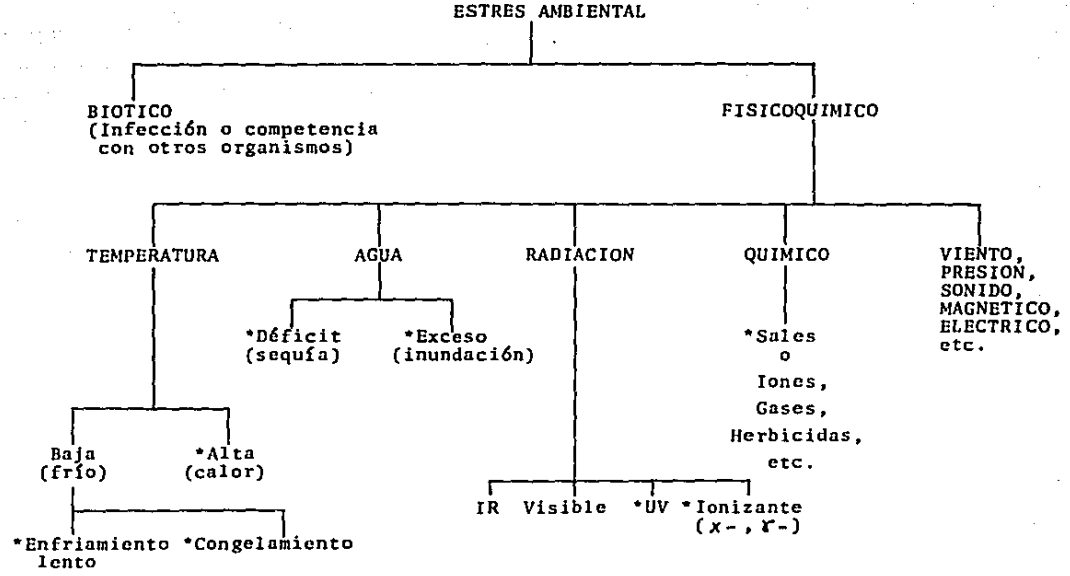

Figura 1. Diferentes tipos de estrés ambiental.

medio de una barrera física (un compuesto químico o una barrera metabólica) que lo aisle o por exclusión del estado estacionario. Al evitar el estrés se evita en consecuencia el posible daño.

La tolerancia es la habilidad de la planta para que aún cuando se establezca el equilibrio con el estrés, no se presente ningún perjuicio en ella. La planta es por tanto capaz de prevenir, disminuir o reparar el daño inducido por el estrés. El evitar el estrés es una adaptación más avanzada, evolutivamente hablando, que la tolerancia; ya que en el primer caso se ha desarrollado un mecanismo que evita el equilibrio y lo reemplaza por el estado estacionario, en el cual la planta no sólo sobrevive sino metaboliza, se desarrolla y completa su ciclo de vida. La tolerancia sólo le permite sobrevivir hasta que el estrés desaparece y entonces, reinicia su metabolismo, crecimiento y desarrollo normales.

4. Estrés de agua.

El estrés de agua puede producirse por una insuficiente o excesiva actividad de agua en el medio ambiente de la planta. En la naturaleza, el primer caso es el resultado de un déficit de agua, la sequía, y es por tanto denominado estrés por déficit de agua, ó acortándolo, simplemente estrés de agua. Cuando una planta es sometida a una pérdida de agua por evaporación artificialmente inducida, se dice que está sometida a un estrés por desecación. Hsiao (1973) propuso que este término se use solamente cuando se haya perdido el 50% ó más de agua de la planta. El estrés osmótico se define como aquél capaz de inducir

una pérdida de agua en el estado líquido.

En tanto que la planta evite el equilibrio con su medio ambiente, no hay límite al estrés de agua al que pueda sobrevivir. Sin embargo, existe un límite definido para la sobrevivencia del daño por deshidratación. Se ha establecido una división de los efectos del estrés de agua en 3 niveles: (1) leve, dado por una baja de varios bars en el potencial de agua de la célula o hasta 8-10% de deshidratación bajo el punto de saturación del tejido; (2) moderado, de -12 a -15 bars o de 10-20% de deshidratación y (3) severo, más de -15 bars. Las deshidrataciones leves y moderadas ocurren dentro de la zona de turgor de la célula, mientras que las severas son aquéllas que ocurren dentro de la zona de flacidez.

En la figura 2 se resumen los efectos de la deshidratación inducida por el estrés de agua. Estos efectos son, per se, elásticos y reversibles. La "única" lesión sería la baja productividad. Si se mantiene por un tiempo crítico o es suficientemente severa por un corto tiempo, la deshidratación reversible da lugar a lesiones verdaderas del tipo directo, indirecto y secundario. Hay 6 diferentes clases de lesiones: inhibición del crecimiento, inanición, acumulación de toxinas, lesiones bioquímicas, salida de iones y deficiencia nutricional; sus relaciones con las posibles causas se describen en la figura 3.

5. Estrés por sales de sodio.

Sólo si la concentración de sales es suficientemente alta

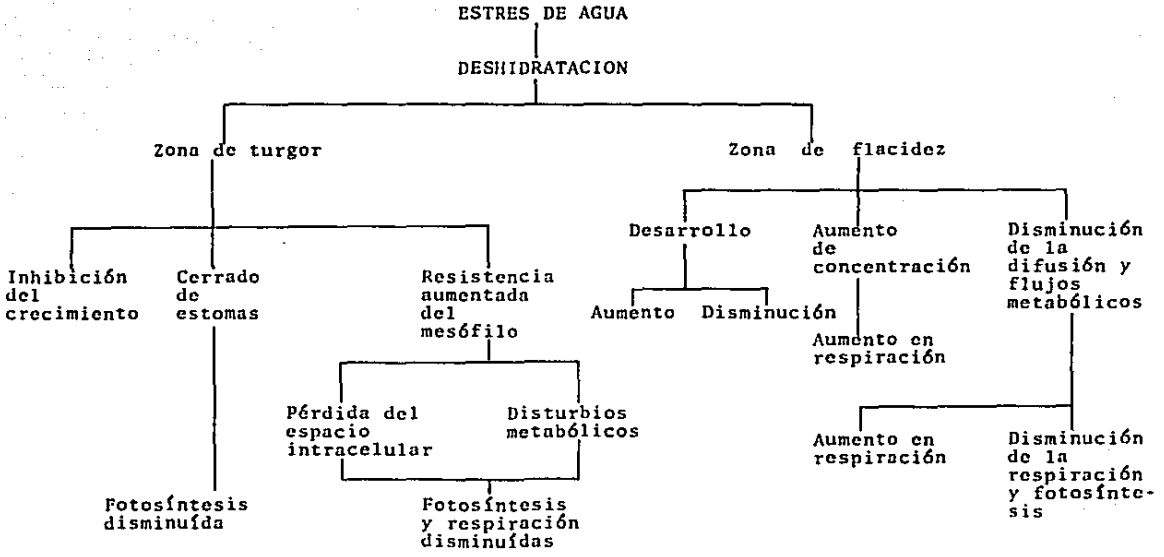

Figura 2. Efectos de la deshidratación, inducida por estrés de agua sobre el crecimiento, la fotosíntesis y la respiración.

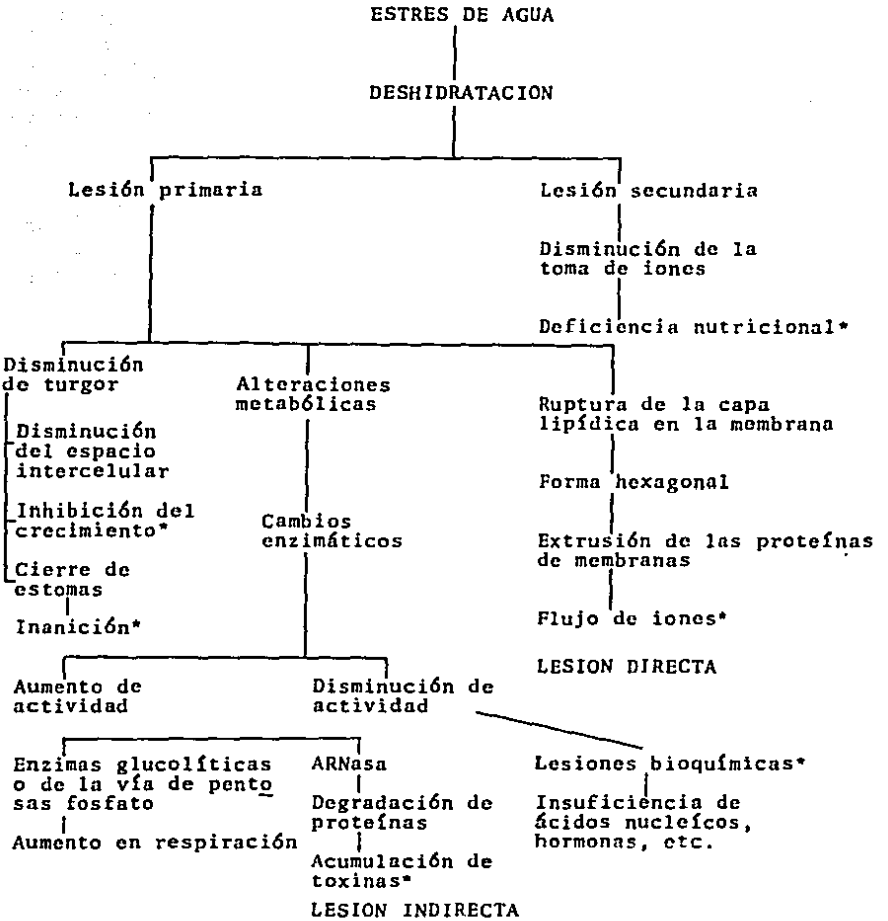

Figura 3. Los seis tipos diferentes de lesiones inducidas por el estrés de agua y sus relaciones con las posibles causas.

para disminuir el potencial de agua de manera importante, entonces se le denominará estrés salino. Si no disminuye el potencial de agua, se habla entonces de un estrés iónico.

Bernstein (1964) clasificó los efectos de la salinidad en osmóticos, nutricionales y tóxicos. En la terminología empleada en el estudio del estrés, los primeros son de tipo secundario, mientras que el último es una lesión primaria ocasionada por la sal.

El estrés osmótico se origina al adicionar sal al agua por lo que disminuye el potencial osmótico; por esto se dice que el estrés salino expone a la planta a un estrés osmótico secundario o estrés fisiológico de sequía.

El estrés nutricional debido a la salinización puede explicarse en base a una supresión de la absorción de nutrientes por la toma de cloruro de sodio en competencia con los iones nutritivos o inclusive a una inversión en el flujo del agua.

Los efectos metabólicos del estrés salino en las plantas se muestran en la figura 4.

6. Metabolismo nitrogenado.

Tanto el nitrato como el nitrógeno atmosférico, dos de las principales fuentes de nitrógeno inórganico disponible para las plantas, deben reducirse hasta amonio antes de que este elemento se incorpore a la materia orgánica. Puesto que los aminoácidos, ya sea libres o unidos en proteínas, son la forma predominante de

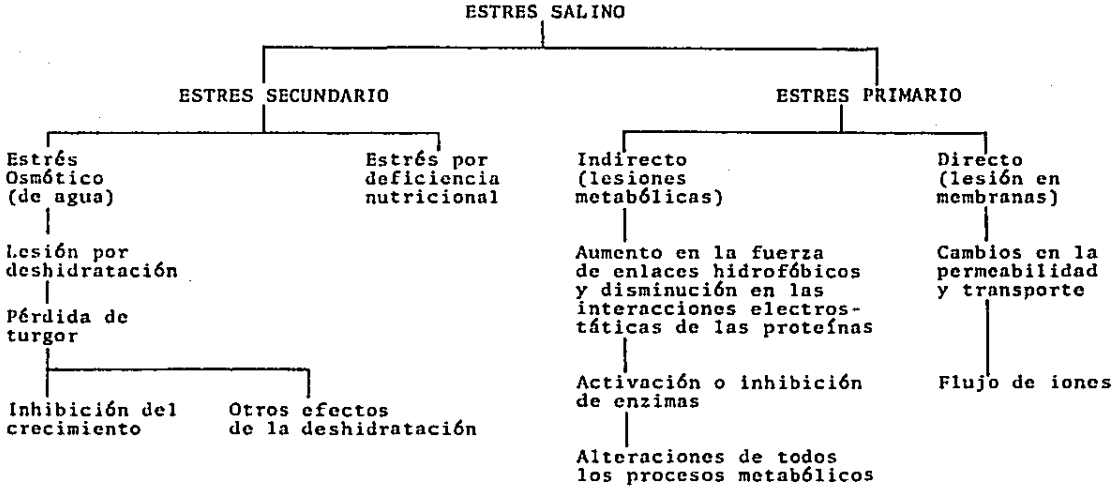

Figura 4. Posibles tipos de lesiones producidas por estrés por sales de sodio.

N orgánico se considera que el producto principal de la asimilación del N en el grupo amino.

Antes de 1970 se consideraba que la ruta principal de entrada del nitrógeno era la vía de la glutamato deshidrogenasa (GDH; EC 1.4.1.4). Esta enzima no sólo está distribuída universalmente sino que también es un mecanismo muy activo para la formación de N α-amino a partir del ión amonio.

$$\alpha\text{-cetoglutarato} + NH_4^+ \xrightarrow{NAD(P)H \quad NAD(P)} \text{glutamato}$$

La otra candidata probable para realizar esta función, en base a su amplia distribución y su gran actividad es la glutamino sintetasa (GS; EC 6.3.1.2) pero aún cuando había formación rápida de glutamina en los tejidos donde se asimilaba amonio, no se conocía el mecanismo por el que el N amido formado durante la asimilación pudiera transferirse a la posición α-amino de los aminoácidos. Sin embargo, Tempest et al (1970) describieron una enzima, la glutamina sintasa, la cual podía catalizar la reacción antes mencionada.

En 1974, Dougall encontró una glutamato sintasa dependiente de NAD(P)H en tejidos no fotosintéticos (EC 1.4.1.13) y Lea y Miflin en el mismo año describieron otra dependiente de ferredoxina en hojas verdes (EC 1.4.7.1). Una característica clave de esta vía es su naturaleza cíclica en la que el glutamato actúa como aceptor y como producto de la asimilación de amonio (figura 5).

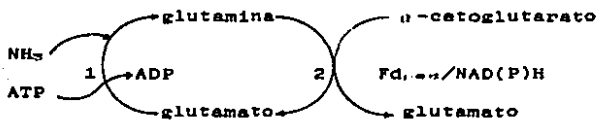

Figura 5. El ciclo GS/GOGAT. 1. glutamino sintetasa; 2. glutamato sintasa.

7. Estrés y metabolismo nitrogenado.

7.1 Fijación de nitrógeno.

Se sabe que la fijación de nitrógeno es un proceso más sensible al estrés que la toma de nitrato (Habish y Mahdi, 1976). El estrés de salinidad disminuyó notablemente la actividad de nitrogenasa en Vigna sinensis, Glycine max y Vicia faba, mientras que en Medicago sativa era capaz de resistir hasta un estrés moderado (80 meq l^{-1} NaCl) (Abdel Wahab y Zahran, 1981). Se ha observado disminución de la fijación de nitrógeno, usando la técnica de reducción de acetileno, en Trifolium repens (Aparicio-Tejo et al, 1980; Engin y Sprent, 1973) y en Lupinus arboreus (Sprent, 1973).

En cuanto al metabolismo nitrogenado en los nódulos de Vigna radiata, hay un incremento en la actividad de GDH cuando son sometidos a un estrés severo de agua. La actividad de la GS disminuye en los casos de estrés moderado y severo de agua (Kaur

et al. 1985). En Medicago sativa solamente la actividad de GOGAT-NADH se ve inhibida durante la sequía y se propone que el ciclo GS/GOGAT está funcionando en los nódulos del control y en aquéllos que recibieron un estrés leve de agua y que a medida que el estrés progresaba se producía un desacoplamiento en este ciclo (Becana et al. 1984).

7.2 Nitrato reductasa.

Una de las enzimas que ha sido estudiada con mayor amplitud es la nitrato reductasa (NR; EC 1.6.6.1) ya que es el paso limitante en la asimilación de nitrato en condiciones de crecimiento adversas.

Generalmente, la actividad de la NR se reduce de manera considerable en plantas sometidas a estrés (Sinha y Nicholas, 1981). Esta disminución puede ser un mecanismo regulatorio, que ha evolucionado con los cambios selectivos de conservación de energía cuando la fotosíntesis se encuentra disminuida o alterada. La regulación de esta enzima, o de cualquier otra, podría estar dada por mecanismos de inactivación/activación, inhibición de la síntesis, degradación o cambio en la especificidad. Puesto que la actividad enzimática no se pierde por completo y además se sabe que puede presentar regulación por inactivación, se puede concluir que el control a través de la inhibición de la síntesis y degradación no es el único mecanismo operante.

Debido a que la acumulación de prolina es uno de los cambios más pronunciados en el metabolismo nitrogenado de plantas

sometidas a estrés, se ha determinado que puede existir una relación entre esta acumulación y la reducción de la pérdida de actividad de la NR. Sinha y Rajagopal (1975) sugieren que la prolina podría "proteger" a la NR de cebada y trigo de la inactivación durante el estrés.

7.3 Acumulación de compuestos nitrogenados y su relación con las enzimas involucradas en la asimilación de amonio.

Una de las respuestas más generalizada de las plantas ante diferentes tipos de estrés es la acumulación de compuestos nitrogenados, entre los cuales destacan prolina y betaína (Stewart y Larher, 1980). Se ha señalado que esta acumulación podría tener varios propósitos, se sugiere que puedan actuar: a) en el mecanismo de osmorregulación; b) como compuestos de reserva de energía y de carbono y nitrógeno reducidos, y c) como estabilizadores de actividades enzimáticas importantes para la sobrevivencia de la planta.

Los aminoácidos: glicina, alanina y prolina y la amina, betaína, no tienen cargas a pH fisiológico y tienen poco efecto sobre la estructura del agua. Se acumulan como solutos compatibles, los cuales deben tener las siguientes características: 1) deben ser muy solubles, por lo que se favorece que sean de bajo peso molecular, 2) no tienen carga neta a pH neutro y 3) deben ser retenidos por la membrana plasmática de la célula contra un gradiente elevado de concentración.

Uno de los papeles metabólicos que se han sugerido para la prolina y la betaína es servir como poza para el nitrógeno de los

compuestos nitrogenados derivados de la pérdida neta de proteína (Savitskaya, 1976). La síntesis de proteína se inhibe inmediatamente por el estrés de agua; sin embargo el proceso de proteólisis no lo está e inclusive está aumentado (Levitt, 1980). Aunque la toma de nitrógeno del medio ambiente está disminuida como consecuencia del estrés, al igual que la actividad de algunas enzimas, el resultado es la acumulación de compuestos nitrogenados solubles de relativamente bajo peso molecular. Es por esto que para llevar a cabo la síntesis de estos compuestos se requiere de la asimilación de novo de amonio y en consecuencia la intervención de enzimas como la glutamato deshidrogenasa (GDH) y el ciclo glutamino sintetasa/glutamato sintasa (GS/GOGAT), las cuales son las vías más importantes para que se lleve a cabo este proceso. Sin embargo, son pocos los reportes que describan como afectan a estas enzimas los diferentes tipos de estrés.

Se ha observado que la GDH aumenta su actividad en Pennisetum americanum, Vigna radiata y Phaseolus vulgaris (Bottacin et al, 1985; Jager y Meyer, 1977; Kaur et al, 1985), mientras que disminuye notablemente en Pisum sativum y Zea mays (Rakova et al, 1978). La actividad de la GS se estimula en plantas resistentes a salinidad de Pisum sativum y Zea mays y la halofita Suaeda maritima y se observa disminución en Vigna radiata (Boucaud y Billard, 1981; Rakova et al, 1978).

HIPOTESIS.

Durante el estrés la toma de nitrógeno de medio ambiente se reduce y existe una pérdida neta de proteína; sin embargo, se presenta una acumulación de compuestos nitrogenados como la prolina y la glicinabetína, por lo que se sugiere que para llevar a cabo la síntesis de estos metabolitos debe haber una asimilación de novo de amonio en la que intervendrán una o ambas vías de asimilación (GDH o el ciclo GS/GOGAT). Si sólo funciona una de ellas, entonces ésta deberá poseer características que le permitan funcionar bajo condiciones de estrés. Por lo tanto la pregunta central es: ¿Cómo se ven afectadas las dos vías de asimilación de amonio (GDH y el ciclo GS/GOGAT) en plantas que han sido sometidas a estrés?.

Uno de los objetivos de este trabajo es determinar si existen diferencias en el comportamiento de estas enzimas entre una planta C_4, Zea mays L. y una C_3, Canavalia ensiformis.

En el primer artículo, se estudiaron: 1) la actividad de las de las enzimas GDH y GS en extractos de plantas de maíz sometidas a estrés de agua o de salinidad; 2) los patrones isoenzimáticos de GDH para las plantas control y las estresadas y 3) la respuesta al estrés in vitro de las enzimas provenientes de una planta sometida a estrés in vivo en comparación con aquéllas de una planta control.

En el segundo artículo, se siguió la misma estrategia para Canavalia ensiformis.

En el tercero, utilizando el sistema de estrés *in vitro*, se estudiaron algunas características de GDH y GS en extractos de plantas de maíz.

OBJETIVOS.

1. Determinar los cambios que se produzcan en las actividades de las enzimas de las vías GDH y GS/GOGAT, en plantas de maíz y de Canavalia ensiformis que serán sometidas a estrés de sequía o salinidad..

2. Determinar y cuantificar si hay acumulación de prolina en los tejidos de plantas estresadas y su relación con la conservación de actividades enzimáticas.

3. Determinar los patrones electroforéticos de las isoenzimas o confórmeros de la GDH, tanto en plantas control como en aquéllas sometidas a estrés.

4. Determinar la respuesta de las enzimas GDH y GS en extractos de plantas sometidas a estrés cuando son sometidas a un segundo choque osmótico con polietilen-glicol in vitro y la acción que tendrían prolina y glicinabetaina en estos sistemas.

MODELOS EXPERIMENTALES.

Se utilizaron dos modelos experimentales: Zea mays L. y Canavalia ensiformis L. (DC), una planta C_4 y una C_3, respectivamente.

Las plantas C_3 y C_4 difieren entre sí en la naturaleza del compuesto que se produce como producto de la fijación del CO_2 durante el proceso fotosintético. En el caso de las C_3, este compuesto es el ácido 3-fosfoglicérico y en las C_4, el ácido oxalacético.

Otra de las diferencias es la presencia del proceso de fotorespiración en las plantas C_3, que ocurre también en las plantas C_4 pero en una proporción mínima en comparación con las anteriores. Durante la fotorespiración, se libera CO_2 y NH_3; éste último compuesto debe ser reasimilado por alguna de las vías ya descritas.

Puesto que existen diferencias en el manejo de esqueletos carbonados, esto podría redundar en diferencias en la asimilación de amonio a compuestos orgánicos en maíz y en C. ensiformis. Por lo que nos propusimos estudiar que es lo que ocurre con los mecanismos de asimilación de amonio cuando estas plantas están sometidas a tratamientos de estrés de agua y de salinidad.

El sistema empleado para imponer los tratamientos de estrés a las plantas fue un sistema hidropónico en el cual las plantas, crecidas durante 10 días sobre agrolita, fueron transferidas a diferentes soluciones: agua para el control; PEG al 41.2% para

aquéllas en estrés de agua (Steuter et al. 1981) y NaCl. 150 ó 300 mM, para inducción del estrés salino (Levitt, 1980), y mantenidas en estas condiciones durante 6 ó 12 h en el caso de maíz ó durante 12 ó 24 h para C. ensiformis.

Se ha mencionado que el PEG puede tener efectos nocivos en las plantas en sistemas hidropónicos como son la absorción de este polímero, reducción de la asimilación y traslocación de fósforo y necrosis de las hojas (Krizek, 1985). Sin embargo, Jager y Meyer (1977) consideran que el PEG es adecuado para la inducción de estrés de agua solamente en períodos cortos.

Por otra parte, los tratamientos para la inducción de condiciones de estrés, i.e. la adición de polietilen-glicol y cloruro de sodio a los sistemas, tienen como resultado final un estrés de agua en ambos casos. En el primero, se da por disminución del potencial de matriz, mientras que en el segundo, esto ocurre por disminución del potencial osmótico.

Para el sistema de estrés in vitro, se utilizó el sistema de Paleg et al (1984). Las concentraciones, tanto de prolina como de glicinabetaina, se eligieron tomando como punto de partida la cantidad máxima acumulada de estos metabolitos en plantas (Levitt, 1980).

METODOLOGIA.

La metodología se encuentra descrita en cada una de las publicaciones.

DISCUSION.

El sitio principal de asimilación de nitrógeno está localizado en las hojas (Pate, 1973) y se asume que los mecanismos involucrados son los mismos descritos para hojas aún cuando este proceso se lleve a cabo en las raíces (Miflin y Lea, 1977). No obstante, se podrían esperar diferencias en el metabolismo relativas a la facilidad o costo de la obtención de reductores, energía o esqueletos carbonados en tejidos fotosintéticos y no fotosintéticos. Hay evidencias que muestran que las diferencias existen también a nivel de los mecanismos que regulan la inducción de enzimas de la asimilación de nitrógeno en raíces y en hojas (Radin, 1975).

Debido a su abundancia, su localización y su baja Km para amonio, la reacción catalizada por la GS probablemente representa la vía principal para la asimilación de amonio en hojas y raíces (Oaks y Hirel, 1985). Sin embargo, la gran actividad de GDH presente en las raíces, comparada con la encontrada en hojas, podría sugerir que bajo condiciones normales la GDH es la vía de asimilación de amonio en las raíces de plántulas de maíz (Loyola-Vargas y Sánchez de Jiménez, 1984). El argumento que se esgrime para no asignar un papel importante a la GDH en la asimilación de amonio ha sido su elevada Km para amonio (Miflin y Lea, 1977; Miflin et al, 1981). Pahlich y Gerlitz (1980) encontraron que la Km era bifásica y dependía de la concentración de NH_4^+ y de iones divalentes presentes en el ensayo, por lo que el argumento de la Km ya no resulta válido.

Se pudo observar que las actividades de la GDH y la GS, provenientes de plantas de maíz y C. ensiformis, responden de manera diferente al estrés dependiendo del tejido del cual provengan los extractos. En las plantas de C. ensiformis el tipo de estrés al que son sometidas marca la diferencia en el comportamiento de las actividades enzimáticas; mientras que en maíz, la duración (intensidad) del estrés es el parámetro crítico.

Los resultados obtenidos de los niveles de actividad de GDH y GS en maíz, sugieren que existe una concertación entre ambas vías de asimilación de amonio: esto es, durante las primeras 6 h de estrés, la vía principal de asimilación de nitrógeno en raíces parece ser la GDH, mientras que en las hojas parece ser el ciclo GS/GOGAT. Durante las siguientes 6 h, el comportamiento se invierte. Se podría entonces especular acerca de la existencia de un tiempo de adecuación que le permite a la planta modificar su metabolismo; de tal manera que si el estrés se prolonga, pueda manejar de manera eficiente sus metabolitos para evitar la carencia de alguno de éstos hasta que las condiciones adversas desaparezcan.

La disminución en la actividad de las enzimas en el control podría deberse a que aún cuando se trató que las condiciones en los sistemas hidropónicos fueran óptimas para las plantas, el cambio de la agrolita a este sistema fue un estrés no deseado que recibieron estas plantas.

En Canavalia ensiformis, Loyola et al (1987) han sugerido

que la GDH es la vía mayoritaria de asimilación de amonio en las raíces de las plantas control. En las plantas que fueron sometidas a estrés salino, tanto GDH como GS presentan la misma tendencia que el control; esto es, no hay cambios con respecto a la vía comúnmente empleada. Sin embargo las plantas sometidas a estrés de agua, utilizan la misma vía que el control (GDH) durante las primeras 12 h para luego cambiar a la vía GS/GOGAT.

En el primer par de hojas, las plantas sometidas a ambos tratamientos de estrés utilizan la vía de la GDH en lugar del ciclo GS/GOGAT como se ha propuesto (Loyola et Al. 1987).

En tejidos de diferentes plantas, se han descrito varias isoenzimas de la GDH (Gil'manov et al. 1967; Nicklisch, 1979; Yue, 1969) y además el patrón de isoenzimas se modifican bajo diferentes condiciones como diferentes fuentes de nitrógeno (Kretovich et al. 1973) o a lo largo del ciclo de crecimiento en cultivos de tejidos de maíz (Loyola-Vargas y Sánchez de Jiménez, 1986), lo que sugiere papeles específicos para cada isoenzima.

Al realizar los barridos de los patrones "isoenzimáticos" se encontró que existen diferencias notables entre los patrones de plantas que no han sido sometidas a estrés con aquéllas que recibieron estos tratamientos, tanto en maíz como en C. angiformis.

Los cambios observados en los patrones isoenzimáticos podrían deberse a: (a) la existencia de una síntesis de novo de "isoenzimas de estrés", aún cuando esta síntesis estuviera disminuída ya que se ha podido observar que uno de los primeros

efectos del estrés es la conversión de polisomas en monosomas (Levitt, 1980) y (b) un rearreglo de las subunidades o modificación de las proteínas ya existentes que conduzcan a cambios en sus actividades catalíticas que les permitan seguir funcionando bajo estas condiciones. Esta modificación podría llevarse a cabo por poliaminas (metabolitos que también se acumulan bajo ciertas condiciones de estrés; Flores et al. 1985), lo que podría estabilizar su estructura manteniendo por tanto su solvatación.

Puesto que la lectura de los barridos de los zimogramas muestran diferencias notables, esto puede sugerir, como ya se dijo, que existan nuevas proteínas o cambios conformacionales o modificaciones de las ya existentes que ahora son menos sensibles al estrés. Para ensayar esta posibilidad se estudiaron las enzimas GDH y GS en los extractos de plantas sometidas a estrés utilizando el sistema in vitro de Paleg et al (1984).

En maíz, no se encontraron diferencias significativas en las actividades de GDH y GS en los extractos de plantas sometidas a estrés de agua o salino por 6 h. Al prolongar el tratamiento hasta 12 h, las actividades de GDH y GS aumentan ligeramente al añadir prolina a los extractos de raíces de plantas con estrés salino; más cuando se adiciona glicinabetaína el incremento se observa en ambos tipos de estrés. En los extractos provenientes de la primera hoja de plantas sometidas a estrés de agua o salino, la actividad de la GS aumenta considerablemente al añadir prolina o glicinabetaína, siendo aún más pronunciada en el último caso.

Sin embargo, en el caso de Canavalia, no se presentan diferencias entre los extractos de raíces y primeras hojas de plantas sometidas a ambos tipos de estrés cuando se tratan con PEG, con PEG y prolina o glicinabetaína.

Aún cuando la acumulación de prolina no alcanza los niveles encontrados en hojas de maíz y cebada (Carceller y Fraschina, 1980; Singh et Al. 1973) sí existe en nuestros sistemas; tal vez esta discrepancia radica en las diferencias entre variedades y especies y principalmente en el grado de deshidratación. Debe tomarse en cuenta que no solamente se acumula prolina (aunque ha sido el metabolito más estudiado), sino también ácido γ-aminobutírico, asparagina, ácido glutámico y glutamina (Hanower y Brzozowska, 1975). La acumulación de glicinabetaína está asociada a características halofíticas y xerofíticas y a habitats áridos y/o salinos (Wyn Jones y Storey, 1981). La Canavalia ensiformis muestra resistencia moderada al estrés de agua y de salinidad comparado con otras especies de leguminosas.

Dado que este segundo tratamiento ya es in vitro entonces el efecto de la prolina y de la glicinabetaína deberá ser producido por un cambio conformacional en las enzimas estudiadas, lo que queda por aclarar es si estas proteínas son las mismas enzimas que las de plantas no estresadas. Aquí cabe la posibilidad de que durante el estrés in vivo se produzcan nuevas proteínas o cambios en las ya existentes y estos se mantengan durante cierto tiempo en la célula, proporcionándole a la planta cierta memoria bioquímica, lo que explicaría porque los sistemas que son sometidos a un segundo estrés responden más rápido y lo resisten

mejor.

Puesto que la adición de polietilen-glicol y la acción "protectora" que ejercen compuestos nitrogenados como prolina y glicinabetaina pueden semejar una situación que tendrían fuerte analogía con las consecuencias de la deshidratación biológica, se decidió que esta estrategia podría proveernos de un sistema en él que pudieramos estudiar cuáles son los cambios que presentan, tanto la GDH como la GS, en una planta sometida a estrés. Es un hecho bien establecido que la solvatación de las proteínas influye en los parámetros termodinámicos de activación de las enzimas (Greaney y Somero, 1979; Low y Somero, 1975) y se podría esperar que cualquier sustancia con la habilidad de mantener la solvatación de las enzimas influira en su actividad. Entre estas sustancias se pueden mencionar al glicerol, sacarosa, hexilen glicol, aminoácidos y sales cuaternarias de amonio (Arakawa y Timasheff, 1982; Gekko y Timasheff, 1981a y b; Lee y Lee, 1981; Paleg et al, 1981 y 1984).

Los resultados del artículo 3 muestran que la adición de PEG a un extracto enzimático de maíz tiene un diferente efecto sobre cada una de las actividades enzimáticas estudiadas: éstas pueden disminuir o mantener un nivel de actividad ligeramente menor al del control, dependiendo del tejido del cual se obtuvo el extracto. Al añadir prolina, puede observarse que en las raíces la actividad de GS aumenta; mientras que en las hojas, esta adición no presenta ningún efecto. Cuando se determinó la Km para amonio de GDH en raíces se observó que en presencia de PEG y prolina, la afinidad por este ión aumentaba 100%.

Estos resultados sugieren que el estrés podría no sólo conducir a la inactivación de enzimas sino también a la inducción de cambios en sus parámetros cinéticos. Por otra parte, es probable que en las raíces de plantas de maíz no se alcance la concentración de 1 M de prolina durante el estrés, pero que la cantidad acumulada sea suficiente para que esta vía resulte funcional. Si esto llegase a ocurrir la planta no se vería en la necesidad de transportar desde las hojas el N α-amino que ha sido asimilado y por tanto, resulta un ahorro de energía y agua que esta asimilación ocurra en las raíces.

Se encontró que tanto el estrés de agua como el salino dan lugar a cambios en las actividades de la GDH y la GS, tanto en maíz como *C. ensiformis*, haciendo que el nitrógeno vaya por una u otra vía dependiendo del tipo de estrés y que la funcionalidad de las vías parece depender de la presencia de isoenzimas o confórmeros más aptos para resistir las condiciones desfavorables a las que se ve sometida la planta.

En resumen, el comportamiento de las enzimas GDH y GS en extractos de plantas sometidas a estrés depende del tejido de donde éstos se obtuvieron y presenta diferencias de acuerdo a la planta: en maíz, el factor crítico es la intensidad del estrés mientras que en *C. ensiformis* es el tipo de estrés. Existen diferencias en los patrones isoenzimáticos de plantas sometidas a estrés, que implican características de resistencia distintas en las enzimas, las cuales fueron detectadas al someter a los extractos de este tipo de plantas a un choque osmótico *in vitro*.

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Study of enzymes involved in ammonia assimilation under water and salt stress in maize

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Abstract

There is still a lot of controversy on how plants can assimilate ammonia during stress. Even though the GS/GOGAT cycle is the most commonly used pathway for this function, there are some reports that point out that GDH may have an important role for incorporating ammonia to amino acids under certain physiological conditions. We have found that the pathway utilized for ammonia assimilation depends on the severity of the stress treatment and on the tissue being studied. It is suggested that during mild stress, GDH is the key enzyme in the roots, while GS functions in the first leaves. The changes observed in activities could be correlated to changes in the isoenzymatic patterns of extracts from stressed and non-stressed plants. These latter changes might be attributed to conformational changes that allow enzymes to remain functional in spite of unfavourable thermodynamic interactions of protein and solutes. A strategy developed by plants to neutralize these effects is the accumulation of nitrogenous compounds, such as proline and glycinebetaine. Our results suggest that there must be a period of adjustment to stress in which these substances are produced, and also that there might be differences in enzyme activities between stressed and non-stressed plants.

Key words: nitrogen assimilation, water stress, salt stress, maize.

Introduction

The vast majority of papers that have been published on the subject of water stress in plants deals with changes at the physiological level and in their metabolism, referring to differences in enzymatic activity levels and the accumulation of certain metabolites that might allow the plants to survive (Hsiao, 1973; Singh, Paleg and Aspinall, 1973; Aspinall and Paleg, 1981; Hanson y Hitz, 1982). There is an increasing number of reports about how a plant can assimilate ammonia during a period of stress but the roles of glutamate dehydrogenase and glutamine synthetase/glutamate synthase cycle are still not clearly defined.

Even though the GS/GOGAT cycle is the most important way of incorporating ammonia into organic compounds (Miflin and Lea, 1980), GDH must not only be assigned a detoxifying role as has been suggested by Loyola-Vargas and Sánchez de Jiménez (1984) and Oaks and Hirel (1985).

During water and salt stress, there is an important accumulation of nitrogenous compounds such as proline and glycinebetaine; the role of these substances is the object of a lot of speculation; they may act as a mechanism of osmoregulation or function as C-, N- and energy storing compounds (Stewart and Lee, 1974; Storey and Wyn Jones, 1977; Aspinall and Paleg, 1981). Another aspect that must be kept in mind is that these compounds can neutralize the unfavourable thermodynamic interactions, which destabilize all macromolecules (Paleg, Stewart and Bradbeer,

1984) and hence, disrupt cellular homeostasis.

The aim of the present study was, to observe the behaviour of the enzymes involved in ammonia assimilation when maize plants were under a treatment of water or salt stress. We also examined the effects of the addition of polyethyleneglycol, proline and glycinebetaine in extracts of these plants, in order to assess if the detected enzymatic activities were in some way different from those in the control extracts.

Materials and methods

Plant material. Maize plants (Zea mays L., var. Chalqueño criollo) were grown in moist agrolite and watered daily for 10 days before harvesting. They were kept in a growth chamber with a 12 hour photoperiod, 30°C during the light period and 25°C in darkness. Water and salt stress (6 or 12 h) were imposed on the plants by changing them to aqueous solutions of polyethyleneglycol (PEG-6000) or sodium chloride: control plants were put in water. The concentration of PEG was 41.2% (equivalent to a water potential value of -2 MPa) and the concentration of NaCl 150 mM. After the stress period was over, plants were harvested: leaves and roots were cut and frozen immediately in liquid nitrogen.

Enzyme extracts. These were obtained as previously described in Miranda-Ham and Loyola-Vargas (1987).

Enzyme assays. NAD-, NADH-GDH and GS assays were performed as described by Loyola-Vargas and Sánchez de Jiménez (1984, 1986). Aminative and deaminative GDH activities were defined as nmoles of cofactor reduced or oxidized per minute per mg protein, respectively and GS activity was expressed as μmoles of Γ-glutamyl-Γ-hydroxamate formed per minute per mg protein.

Protein content. Protein was determined according to Peterson (1977) using bovine serum albumin as a standard.

PEG-induced precipitation. Precipitation with PEG was performed as described in Miranda-Ham and Loyola-Vargas (1987).

Isoenzymatic determinations. Separation was performed using Davis' discontinuous electrophoretic system (Davis, 1964) and activity was developed using the following mixture: 125 mg sodium glutamate, 25 mg NAD, 12.5 mg of 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyltetrazolium bromide and 2.5 mg of phenazine methosulfate in 25 ml 100 mM Tris-HCl pH 9.4.

Results

NADH-GDH activity from roots of plants subjected to water or salt stress for 6 h did not differ much to that found in the control. However, after a longer period of stress (12 h) this activity diminished rapidly (Figure 1A). Meanwhile, GS activity did not decrease so markedly; after 12 h, 70 and 90% of the initial activity remained in water and salt stressed plants, respectively (Figure 1B).

The results obtained for these enzymes in the first leaves are shown in figure 2. NADH-GDH in stressed plants diminished with time, but to a lesser extent than in the control plants (figure 2A). GS activity (Figure 2B) was surprisingly high when plants were stressed for 6 h in both types of stress but it returned to control levels after 12 h.

Since it has been suggested that NADH- and NAD-GDH activities are located in the same protein, and several isoenzymes have been described, the changes that were found in the enzymatic activities might reflect changes in the isoenzymatic patterns for GDH in stressed and non-stressed plants. The isoenzymatic patterns of these plants were determined using a discontinuous electrophoretic system and GDH activity was developed by an *in situ* reaction. In figure 3a, two activity peaks can be observed in the zymograms of root extracts from non-stressed plants; while in stressed plants the pattern has changed (Figures 3B and 3C), peak III has appeared in extracts of roots from plants under water (Figure 3B) and salt (Figure 3C) stress for 6 h. In the 12 h treatment for the water stressed plants, the activity was very low. In the salt stressed plants, although activity was lower than at 6 h, a new pattern, with peaks Ia, Ib and III was detected.

In figure 4, the zymograms with the extracts of first leaves of non-stressed plants (A), water (B) and salt (C) stressed plants are shown. In the first case, three activity peaks were observed, contrasting with the two peaks found in the roots. In plants under water- and salt-stress treatment for 6 h, only peak

II appeared. In th 12 h treatments, peaks Ia, III and IIIa were observed in water stressed plants and only peak IV was detected in salt-stressed plants.

In order to assess the behaviour of the enzymes in extracts of non-stressed and stressed plants when treated in vitro with different concentrations of PEG and/or proline and glycinebetaine, we employed the system described by Paleg and coworkers (1984). The concentration used for both compounds was 1 M. As may be seen in figures 5A, 5B and 5C, NADH-GDH activities from the three extracts presented the same pattern, with or without the addition of proline or glycinebetaine. GS activity of roots from salt stressed plants differed from both the control and the water stressed ones: it increased despite the action of PEG. When proline or glycinebetaine were added, this increment was not observed. Although GDH and GS activities did not differ markedly in the extracts from first leaves of the three treatments when PEG and/or proline and glycinebetaine were added, the activity levels of these enzymes were lower than those observed in roots (Figure 6).

After the stress treatment has been prolonged up to 12 h, a differential action of proline and glycinebetaine was observed. GDH and GS activities of extracts from roots of salt stressed plants (Figures 7B and 7D) were more stable after the addition of proline compared to those from water stressed plants. No such difference could be observed with glycinebetaine (Figures 7C and 7F).

Discussion

Although there has not been an agreement on which is the main pathway for ammonia assimilation to amino acids during stress conditions, we have observed that in maize plants subjected to water or salt stress, there seems to be a combination of the GS/GOGAT cycle and GDH pathway. In the 6 h treatment, GDH activity in the roots is maintained at the same level as in the control, while GS activity decreases slightly. As the stress period continues, the reverse situation could be observed; approximately 80% of GS activity was maintained and GDH diminished rapidly.

GDH activity in the first leaves drops through out the stress treatment. Meanwhile, there is a five- to six-fold increment in GS activity level as has been observed in Suaeda leaves (Boucaud and Billard, 1979). After 12 h of stress treatment, both activities were at the same level as in the control.

PEG treatment can be used for inducing water stress but it must be noted that its effects are more severe than inducing this stress by simple witholding water (Jager and Meyer, 1977). Hence, it may be suggested that during mild stress, GDH is the key enzyme for ammonia assimilation in the roots, whereas GS is functioning in the first leaves. As may be seen from our results, the pathway utilized for ammonia assimilation differs greatly depending on the severity of the stress treatment and on the tissue that is being studied.

The changes observed in the zymograms could be attributed to a number of factors: *de novo* synthesis of new isoenzymes, rearrangement of subunits or modification of pre-existing enzymes that lead to alterations in their catalytic and regulatory properties. The latter is the most favoured one in relation to proteins involved in nitrogen metabolism, since conformational changes that diminish the deleterious effects of dehydration are very important for enzyme activity (Huber, 1974; Kramer, 1974; Low and Somero, 1975; Miranda-Ham and Loyola-Vargas, 1987).

When the *in vitro* system described by Paleg *et al* (1984) was employed to assess the effects of an osmotic shock induced by PEG, and the protection against inactivation that might exert proline and glycinebetaine, no significant differences in the behaviour of GDH and GS could be detected in extracts from roots and first leaves of plants under water and salt stress treatment for 6 h. After prolonging the treatment for another 6 h, there were some differences in the activities of extracts from stressed plants compared to those of non-stressed ones. In roots, GDH and GS activities were very similar in the three treatments but upon addition of proline there was a slight increase in these activities in extracts of salt stressed plants. When betaine was added, the increment was observed in both types of stress. GS activity in extracts form first leaves of salt stressed plants showed a marked increase when either proline or glycinebetaine were added, being more pronounced in the latter case.

Our results suggest that there must be a period of adjustment to stress conditions. During this period synthesis of

proline occurs, reaching a higher level after 12 h (data not shown) and thus enzymatic activities remain functional and may even be stimulated. Proline does accumulate in the stressed plants, and although the levels were much lower than those observed in barley (Singh *et al.* 1973; Carceller and Fraschina, 1980), *de novo* nitrogen assimilation was necessary after this synthesis. In conclusion our results show that both pathways of nitrogen assimilation, GS/GOGAT and GDH, were used, depending on both the type of stress and the tissue of the plant in which nitrogen assimilation occurs.

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Legends to figures

Figure 1. NADH-GDH (A) and GS (B) activities in roots from non-stressed (□), water (○) and salt (△) stressed maize plants.

Figure 2. NADH-GDH (A) and GS (B) activities in the first leaves from non-stressed (□), water (○) and salt (△) stressed maize plants.

Figure 3. Scannings of GDH zymograms from extracts of roots from non-stressed (A), water (B) and salt (C) stressed plants. Light lines correspond to the 6 h stress treatment, and heavy lines to the 12 h treatment.

Figure 4. Scannings of GDH zymograms from extracts of the first leaves from non-stressed plants (A), water (B) and salt (C) stressed plants. Light lines correspond to the 6 h treatment and heavy lines to the 12 h treatment.

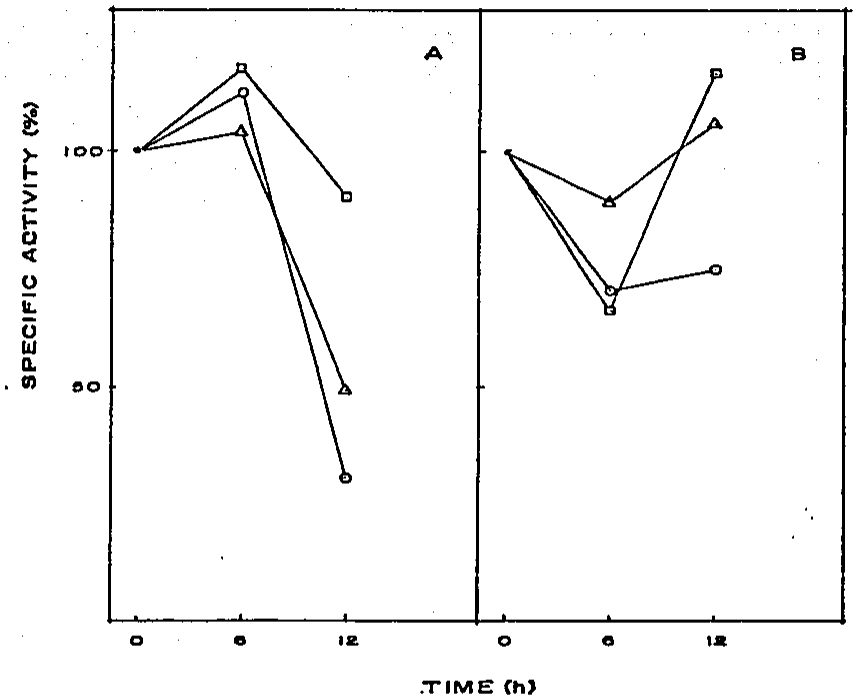
Figure 5. NADH-GDH and GS activities in root extracts from non-stressed (□), water (○) and salt (△) stressed plants for 6 h and then subjected to *in vitro* treatment with PEG (A,D), PEG and proline (B,E) or glycinebetaine (C,F).

Figure 6. NADH-GDH and GS activities in first leaves extracts from non-stressed (□), water (○) and salt (△) stressed plants for 6 h and then subjected to *in vitro* treatment with PEG (A,D), PEG and proline (B,E) or

glycinebetaine (C,F).

Figure 7. NADH-GDH and GS activities in root extracts from non-stressed plants (□) and plants under water (○) and salt (△) stress for 12 h and then subjected to an *in vitro* treatment with PEG (A,D), PEG and proline (B,E) or glycinebetaine (C,F).

Figure 8. NADH-GDH and GS activities in first leaves extracts from non-stressed plants (□) and plants under water (○) and salt (△) stress for 12 h and then subjected to an *in vitro* treatment with PEG (A,D), PEG and proline (B,E) or glycinebetaine (C,F).



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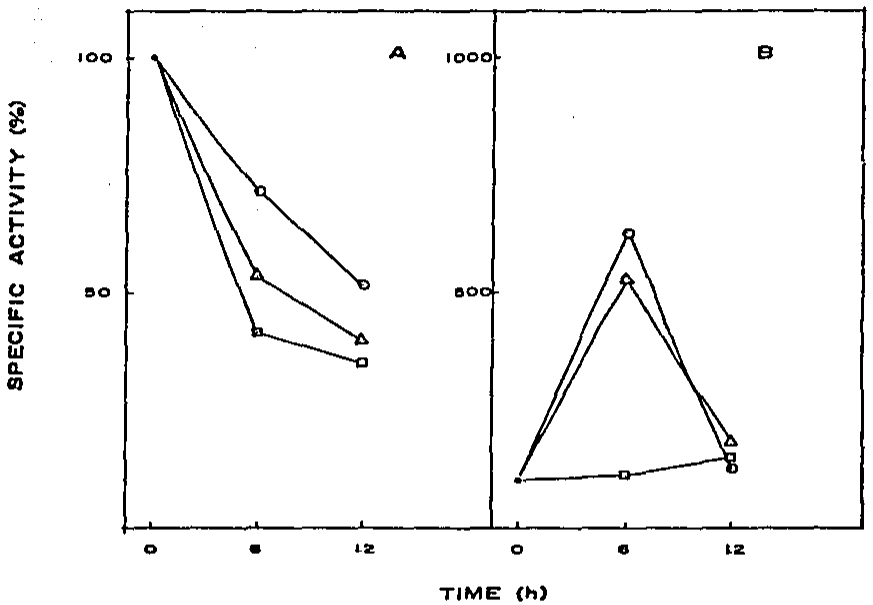

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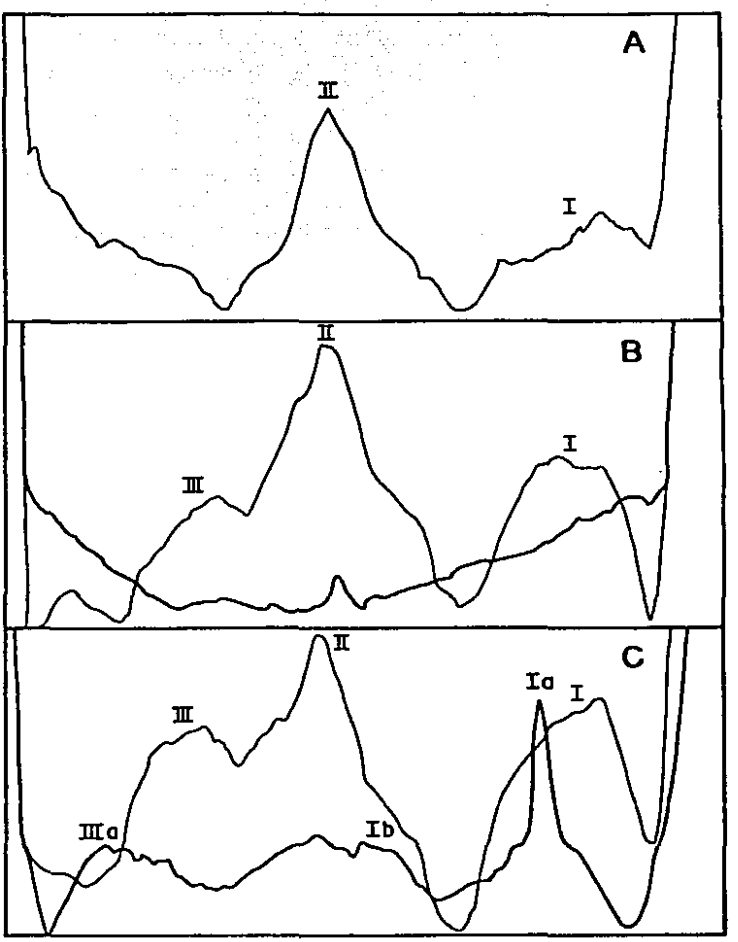

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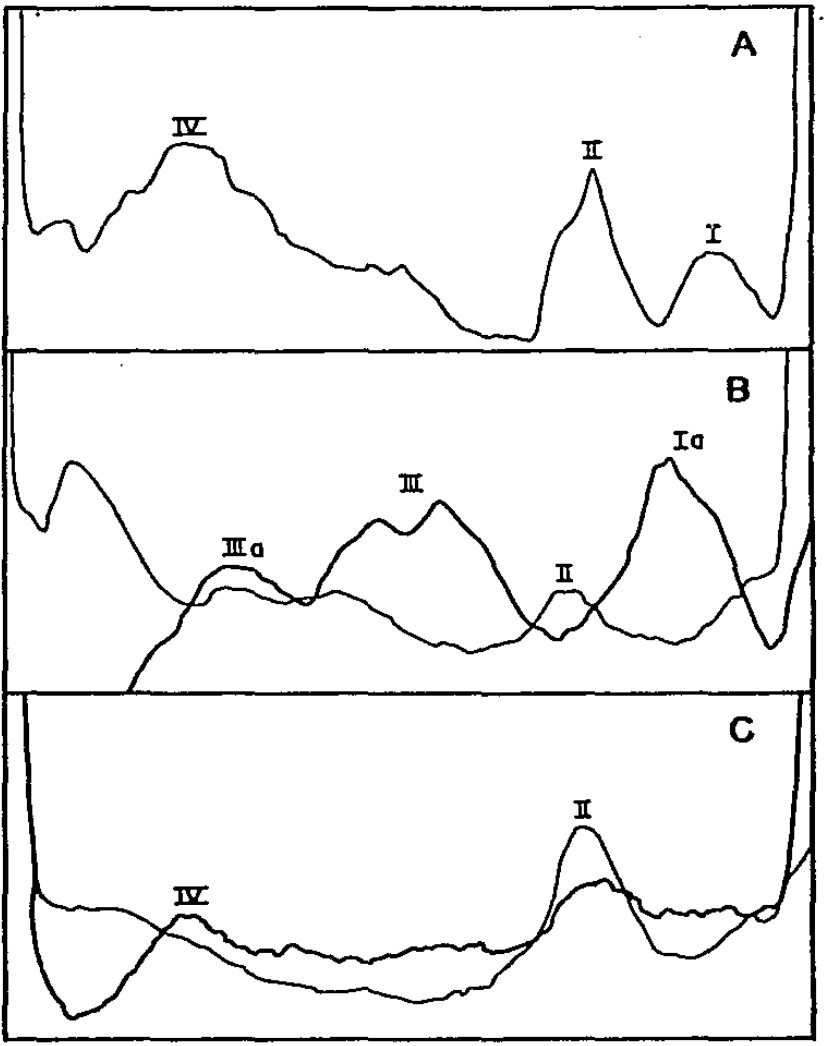

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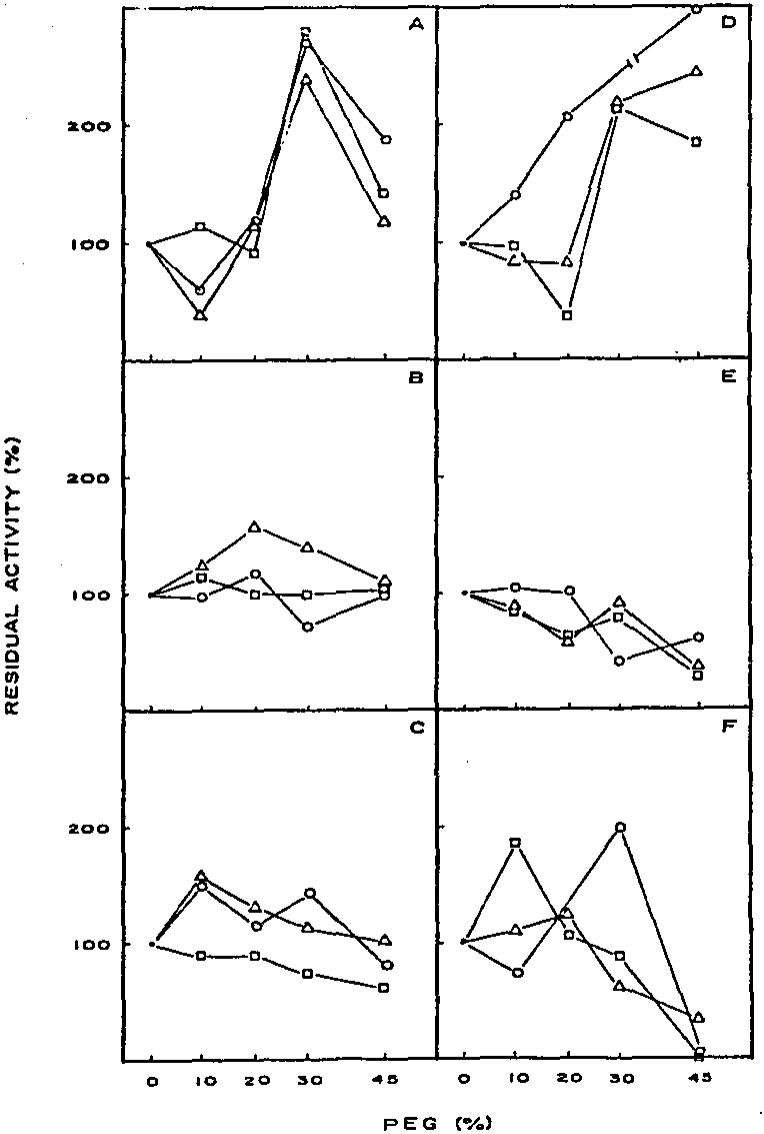

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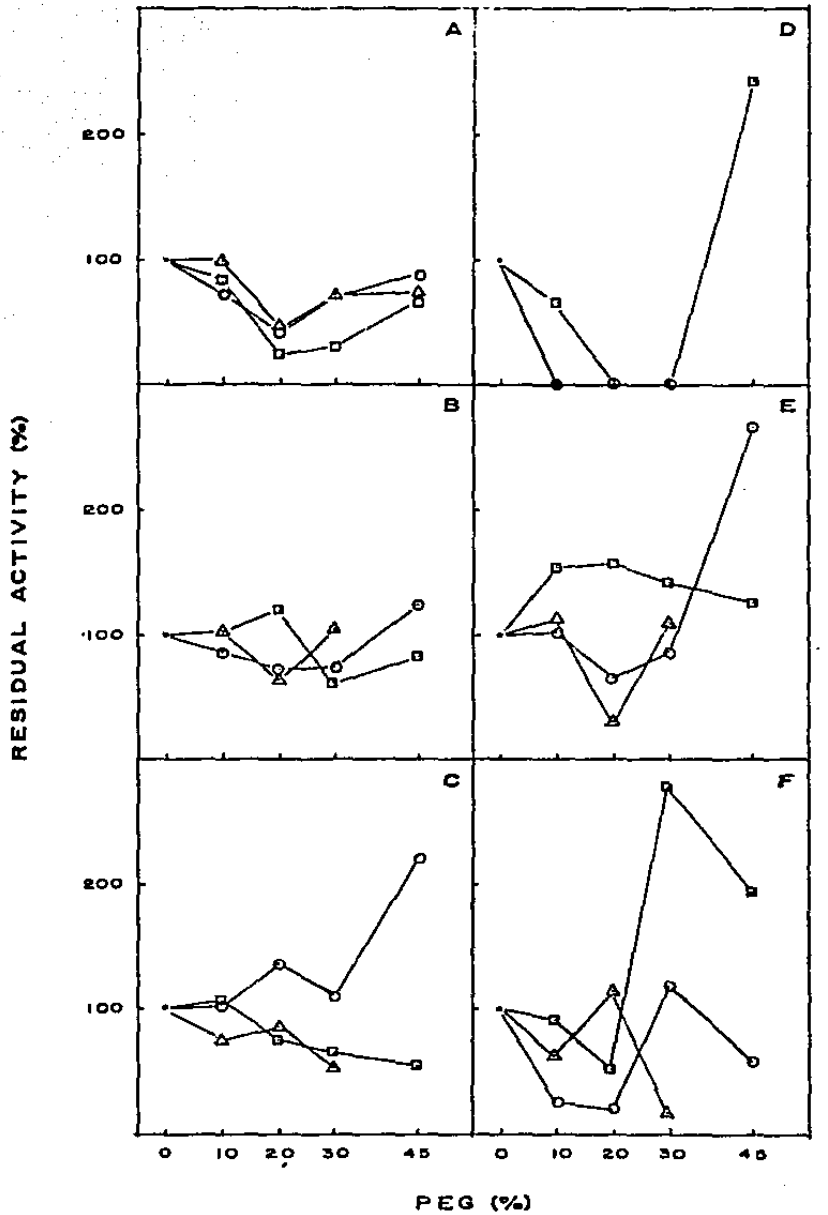

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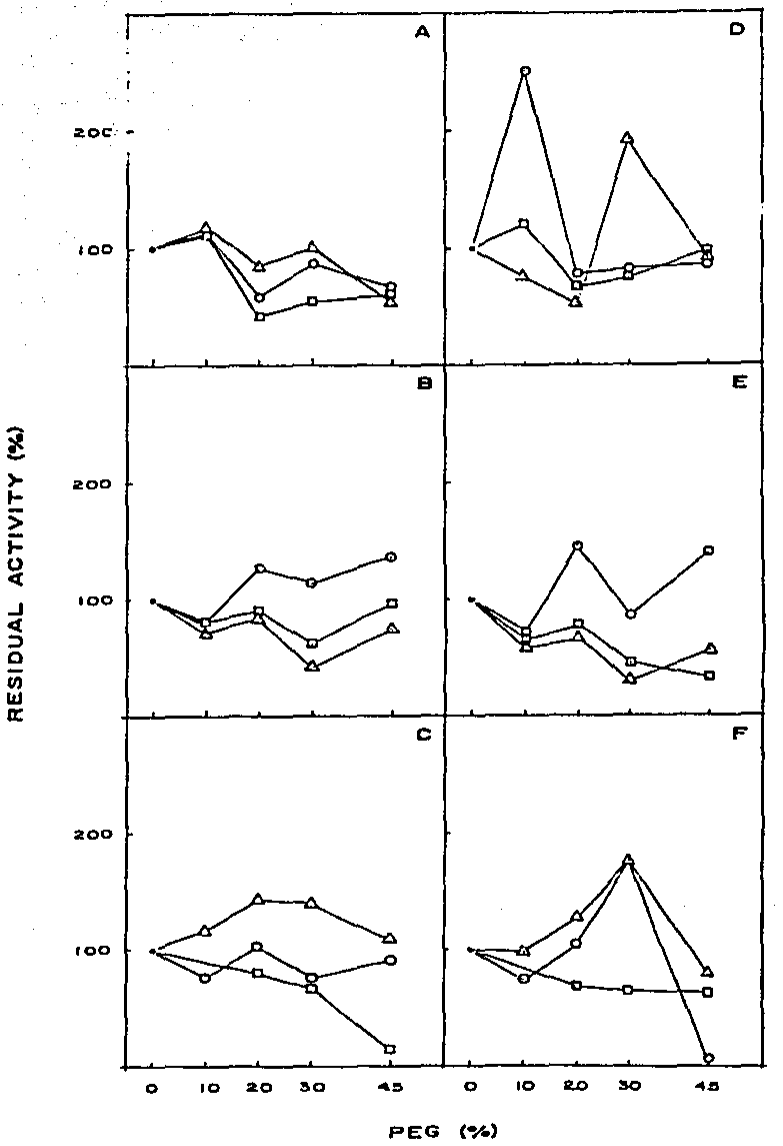
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Figure 7.

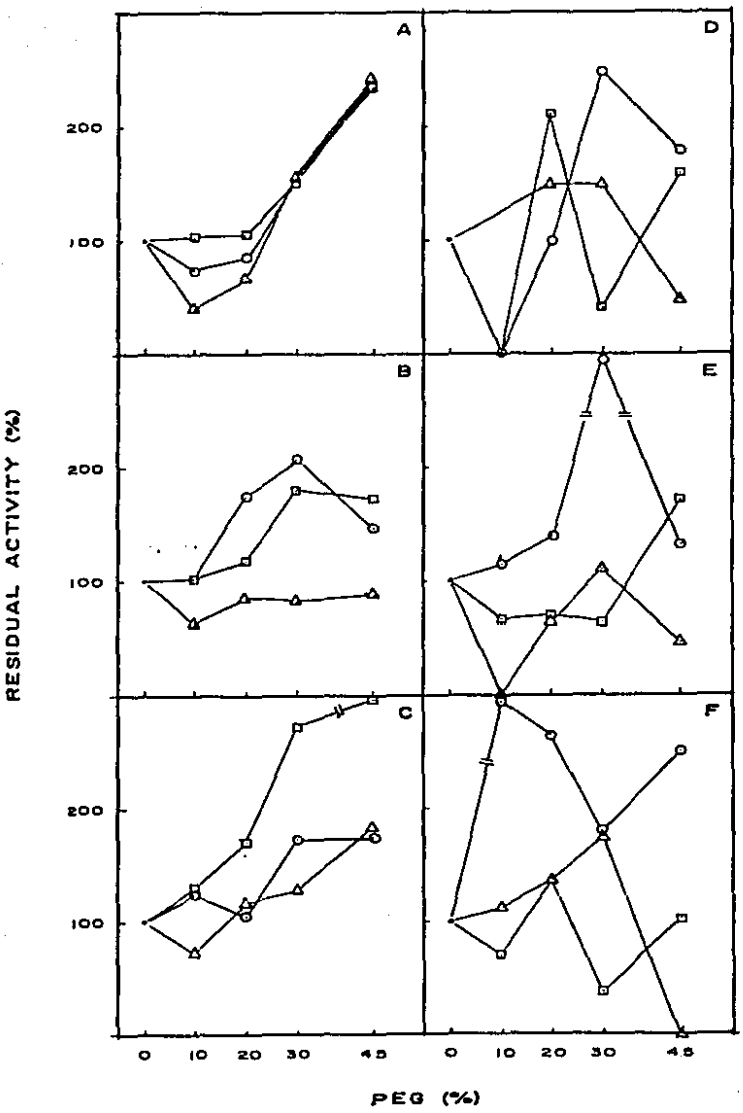

Figure 8.

Sometido a Plant and Cell Physiology.

Ammonia assimilation in *Canavalia ensiformis* plants under water and salt stress.

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Number of figures: eight

Ammonia assimilation in *Canavalia ensiformis* plants under water and salt stress.

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Abstract

Nitrogen fixation and ammonia assimilation in nodules are the two subjects that have been thoroughly studied under stress conditions. In contrast, scarce information is available on the behaviour of enzymes involved in ammonia assimilation to organic compounds in plants of the Leguminoseae family subjected to stress. In some cases, such as it is in Canavalia ensiformis, the pathway(s) utilized for ammonia assimilation in normal conditions are still not clearly defined. Loyola-Vargas et al. (J. Plant Physiol. 1987, in press) have suggested that during early seedling growth, glutamate dehydrogenase and asparagine synthetase are fundamental for ammonia assimilation but when the plant acquires full photosynthetic activity, the GS/GOGAT cycle is the preferred pathway for this process in aerial parts. We found that under stress conditions C. ensiformis plants switch to the alternative pathway of assimilation depending on the nature of the stress and the tissue in which this process takes place.

Key words: Ammonia assimilation, Canavalia ensiformis, Leguminosae, stress.

Introduction

L-canavanine (2-amino-4-guanidoxybutyric acid) is an important nitrogen storing metabolite in at least 500 species of the Leguminoseae family (Bell et al., 1978). In C. ensiformis the mobilization and utilization of nitrogen through L-canavanine occurs via arginase which cleaves this compound to L-canaline and urea. Urea is hydrolized by urease to CO_2 and NH_3. It has been proposed that the latter is assimilated and incorporated into the amide nitrogen of asparagine (Rosenthal, 1970). Loyola-Vargas et al. (1987) suggest that in the early stages of seedling growth aspargine synthetase and glutamate dehydrogenase both play a fundamental role in the reassimilation of ammonia in C. ensiformis; however, when the plant acquires full photosynthetic capacity GS/GOGAT becomes the principal pathway of reassimilation in the aerial parts of the plant.

On the other hand, C. ensiformis shows a moderate resistance to water and salt stress compared to other species of legumes. Once established, it can resist drought conditions on behalf of its radicular system.

Studies on nitrogen metabolism during stress conditions in this family have been centered on nitrogen fixation (Aparicio-Tejo et al. 1980; Abdel Wahab and Zahran, 1981; Randall Weisz et al., 1985) and nitrogen metabolism in root nodules (Becana et al., 1984; Kaur et al., 1985); but reports on the enzymes involved in ammonia assimilation to organic compounds are very scarce.

The objetive of the present study was to determine the behaviour of enzymes involved in ammonia assimilation when salt or water stress treatments were imposed to C. ensiformis plants. We also examined, using the in vitro system of Paleg et al. (1984) the effects of polyethyleneglycol, proline and glycinebetaine on GDH and GS activities of extracts from non-stressed and stressed plants.

Materials and Methods.

Plant material. Canavalia ensiformis plants were grown in moist agrolite and watered daily for 10 days before harvesting. They were kept in a growth chamber with a 12 hour photoperiod, 30°C during the ligth period and 25°C in darkness. Water and salt stress (12 or 24 hours) were imposed on the plants by changing them to aqueous solutions of polyethyleneglycol (PEG 6000) and sodium chloride; control plants were put in water. The concentration of polyethyleneglycol was 41.2% (equivalent to a water potential value of -2 MPa) and the concentration of sodium chloride, 300 mM. After the stress period was over, plants were harvested; leaves and roots were cut and frozen immediately in liquid nitrogen.

Enzyme extracts. These were obtained as has been previously described by Miranda-Ham and Loyola-Vargas (1987a).

Enzyme assays. NAD-, NADH-GDH and GS assays were performed as described by Loyola-Vargas and Sánchez de Jiménez (1984, 1986). Aminative and deaminative GDH activities were defined as nmoles

of cofactor (reduced or oxidized) per minute per mg protein, respectively and GS activity was expressed as μmoles of γ-glutamyl-γ-hydroxamate formed per minute per mg protein.

Protein content. It was determined according to Peterson (1977) using bovine serum albumin as a standard.

PEG-induced precipitation. It was performed as described by Miranda-Ham and Loyola-Vargas (1987a).

Isoenzymatic determinations. Separation was performed using a discontinuous electrophoretic system (Davis, 1964) and activity was developed using the following mixture: 125 mg sodium glutamate, 25 mg NAD, 12.5 mg 3-(4,5-dimethylthiazolyl-2-)-2,5-diphenyltetrazolium bromide and 2.5 mg of phenazine methosulphate in 25 ml 100 mM Tris-HCl pH 9.4.

Results.

NADH-GDH activities detected in the roots of non-stressed and salt-stressed plants diminished gradually; in the case of water-stressed plants, this activity diminished faster during the first 12 h of treatment (figure 1A). Contrastingly, GS activity in roots from water-stressed plants showed an important decrease, followed by an increment up to 110%. In roots from salt-stressed plants, there was a constant decrease in GS activity through out the period of treatment.

The results obtained for these enzymes in the first pair of leaves are shown in figure 2. NADH-GDH activity in water-stressed

plants did not show significant changes in any period of treatment; whereas in salt-stressed plants this activity increased up to the same level as in the water-stressed ones. GS activity (figure 2B) in salt-stressed plants decreased markedly. Meanwhile, GS activity observed in water-stressed plants diminished less dramatically than in the controls.

It has been demonstrated in a number of plants that the isoenzymatic pattern of GDH changes when they are subjected to different treatments; therefore, we determined the isoenzymatic patterns of non-stressed and stressed plants. Determinations were performed using a discontinuous electrophoretic system and GDH activity was developed by an *in situ* reaction. In figure 3A and 3C, only one activity peak could be observed in root extracts from non- and salt-stressed plants; while in water-stressed plants, peak II could be detected in addition to peak I (figure 3B). In the 24 h treatment for stressed plants, though activity was lower in both cases, peak I could also be observed as well as peak Ia for water- and peak II for salt-stressed plants.

Zymograms from first leaves extracts of non- (A), water- (B) and salt- (C) stressed plants are shown in figure 4. In the case of control plants, we observed that there was a single peak (peak I) as has been seen for root extracts. Even though their Rf's are similar, their activities in this extract were lower. For the water-stressed plants, isoenzymatic pattern obtained for the 12 h treatment was similar to that obtained for the longer one; i.e. two peaks were observed (peaks I and II) but their activity ratios varied with time. In plants under salt stress treatment,

peak Ia appeared in the first 12 h; when this treatment was prolonged up to 24 h, the pattern changed completely: peak I had almost disappeared while peaks Ib and Ic comprised all the detected activity. It must be remarked that in the case of first leaves extracts from 24 h stressed plants, activity was not only lower than in the 12 h ones but it increased as may be seen for the salt-stressed plants.

In order to assess the behaviour of the enzymes in extracts of non-stressed and stressed plants when treated in vitro with different concentrations of PEG and/or proline and glycinebetaine, we employed the system reported by Paleg and coworkers (1984). The concentration of proline and glycinebetaine was 1 M. As may be seen in figure 5, NADH-GDH activities from the three root extracts showed the same pattern when PEG was added. These activities were more stable upon addition of proline or glycinebetaine to the system. The same behaviour could be observed for GS activities in root extracts (figures 5D, 5E and 5F) and in first leaves extracts (figures 6A, 6B and 6C). No GS activity could be detected in first leaves extracts from salt-stressed plants when PEG or PEG plus glycinebetaine were added to the system (figures 6D and 6F). This activity was slightly more stable with the addition of proline (figure 6E). In the case of extracts from water-stressed plants, GS activity increased when proline was present but not with glycinebetaine. GDH and GS activities did not differ markedly in the extracts from first leaves of the three types of treatment (24 h) when PEG and proline or glycinebetaine were added (figure 8). These activities

showed to be more stable in extracts of roots from stressed plants as may be seen in figures 7A and 7D. When PEG and proline were present GS activity from extracts of salt-stressed plants increased, but this increment was more pronounced when glycinebetaine was added (figure 7F).

Discussion.

It has been suggested (Loyola-Vargas et al., 1987) that GDH is the key enzyme involved in ammonia assimilation in roots from non-stressed plants. we have observed that in salt-stressed plants GDH activity is maintained at the same level as in the control through out the treatment while GS decreases rapidly. For water-stressed plants, the situation is reversed: even though GS activity diminished in the same rate as in salt-stressed plants in the 12 h treatment, it increased in the following 12 h.

In the case of the first leaves, the stressed plants utilized the GDH pathway instead of the GS/GOGAT cycle for ammonia assimilation since GS activity decreased dramatically for water-stressed ones while GDH is at the same level as in the control. GDH activity in salt-stressed plants increased 60% respect to the control level in the first 12 h of treatment. After 24 h of stress, GDH activity in water- and salt-stressed plants were similar. Our results indicate that the pathway utilized for ammonia assimilation differs greatly depending on the nature of the stress treatment and on the tissue that is being studied.

The changes observed in the zymograms are in agreement with the activity levels detected in the roots and first leaves of non-stressed and stressed plants. The root extracts from salt-stressed plants showed only one peak of similar dimensions as in the control and in the case of extracts of water-stressed plants, the peak is smaller and GDH activity is lower. Furthermore, peak I activity observed in first leaves extracts from water-stressed plants is higher than the same activity in salt-stresed plants but the latter is similar to that found in the control. The changes in the isoenzymatic patterns could be attributed to a rearrangement of subunits or modification of pre-existing enzymes that lead to alterations in their catalytic and regulatory properties (Miranda-Ham and Loyola-Vargas, 1987b).

We found that no significant difference in the behaviour of GDH and GS could be detected in extracts from roots and first leaves of plants under water and salt stress treatment for 12 or 24 h, when the in vitro system of Paleg et al. (1984) was employed for assessing the effects of an osmotic shock induced by PEG. It has been suggested that nitrogenous compounds, such as proline and glycinebetaine, might exert a protective action against enzyme inactivation during stress conditions (Paleg et al., 1984; Miranda-Ham and Loyola-Vargas, 1987a). When we added these compounds to the three types of extracts treated with different concentrations of PEG, GDH and GS activities both were more stable than those in extracts that were not supplemented with proline or glycinebetaine. There was not a differential protective response as has been observed for maize (Miranda-Ham

and Loyola-Vargas, 1987b).

The accumulation of nitrogenous compounds has been observed in a number of plants as a response to water and salt stress (Stewart and Larher, 1980; Aspinall and Paleg, 1981) and even though C. ensiformis accumulated proline, this plant did it in a lesser extent that in the cases mentioned above (data not shown). Since C. ensiformis shows a moderate resistance to the stress treatments that were applied, it may be suggested that a longer period of stress might be necessary for triggering proline's accumulation.

In summary, we have found that C. ensiformis plants subjected to stress switch from one pathway of ammonia assimilation to the alternative one depending on the nature of the stress treatment and the tissue where this assimilation takes place.

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Legends to figures

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Figure 2. NADH-GDH (A) and GS (B) activities in the first leaves of non-stressed (□), water (○) and salt (△) stressed plants.

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Figure 7. GDH and GS activities in root extracts from control (□), water (○) and salt (△) stressed plants for 24 h and then subjected to an *in vitro* treatment with PEG (A,D), PEG and proline (B,E) or glycinebetaine (C,F).

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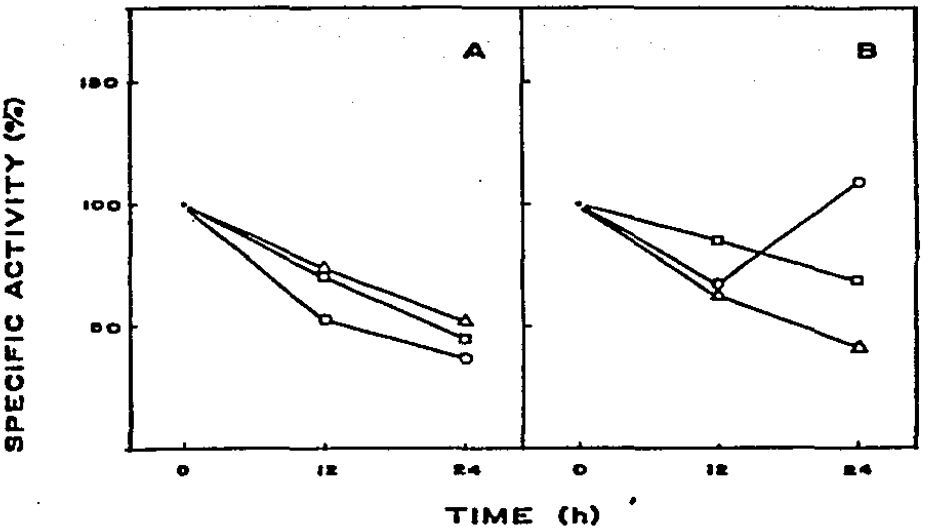
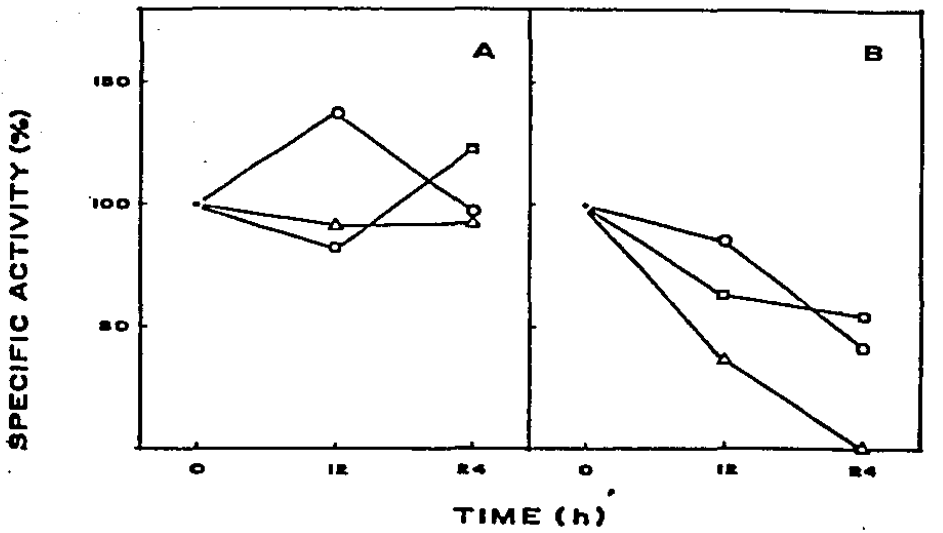


Figure 1.



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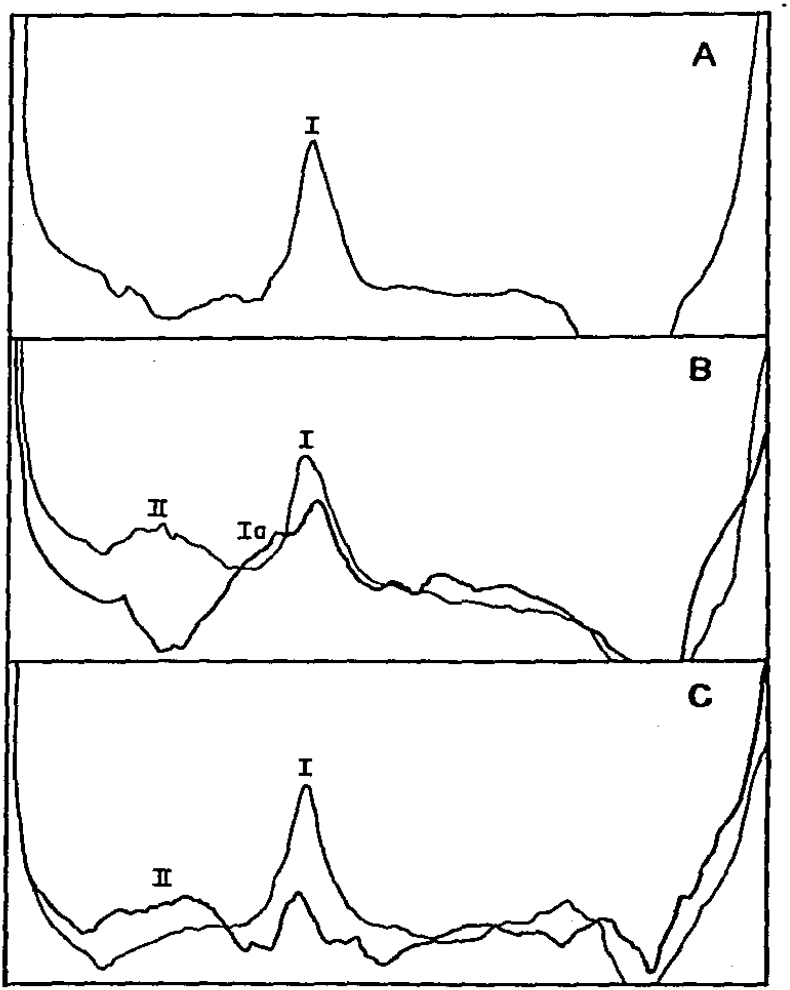

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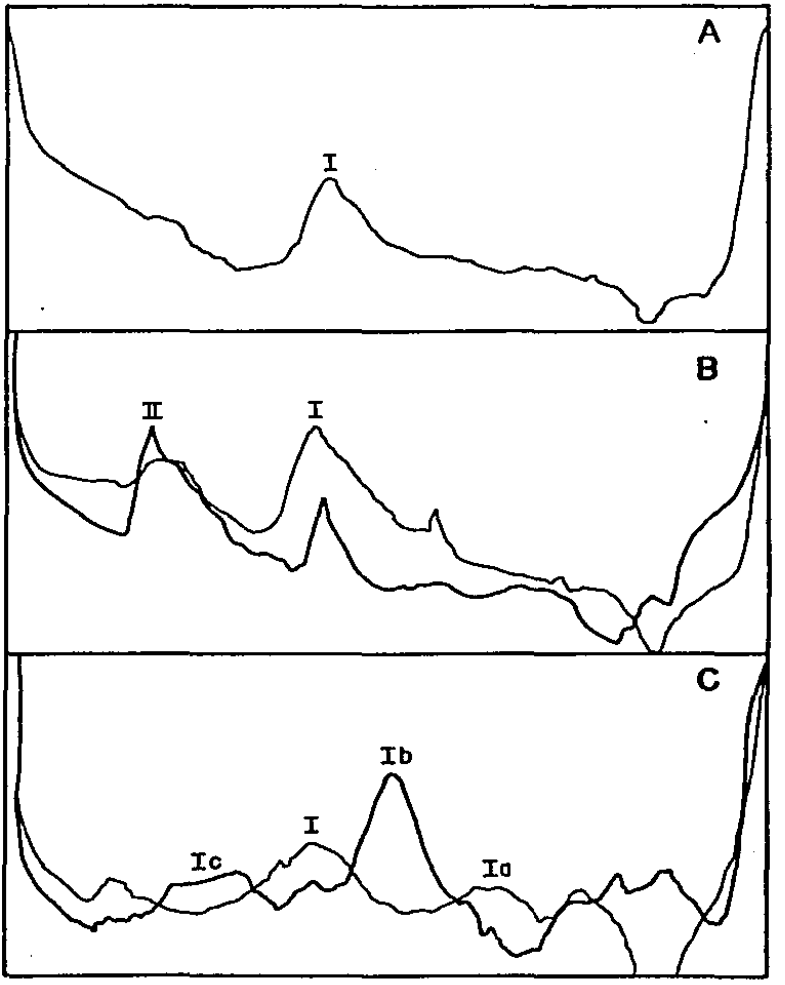

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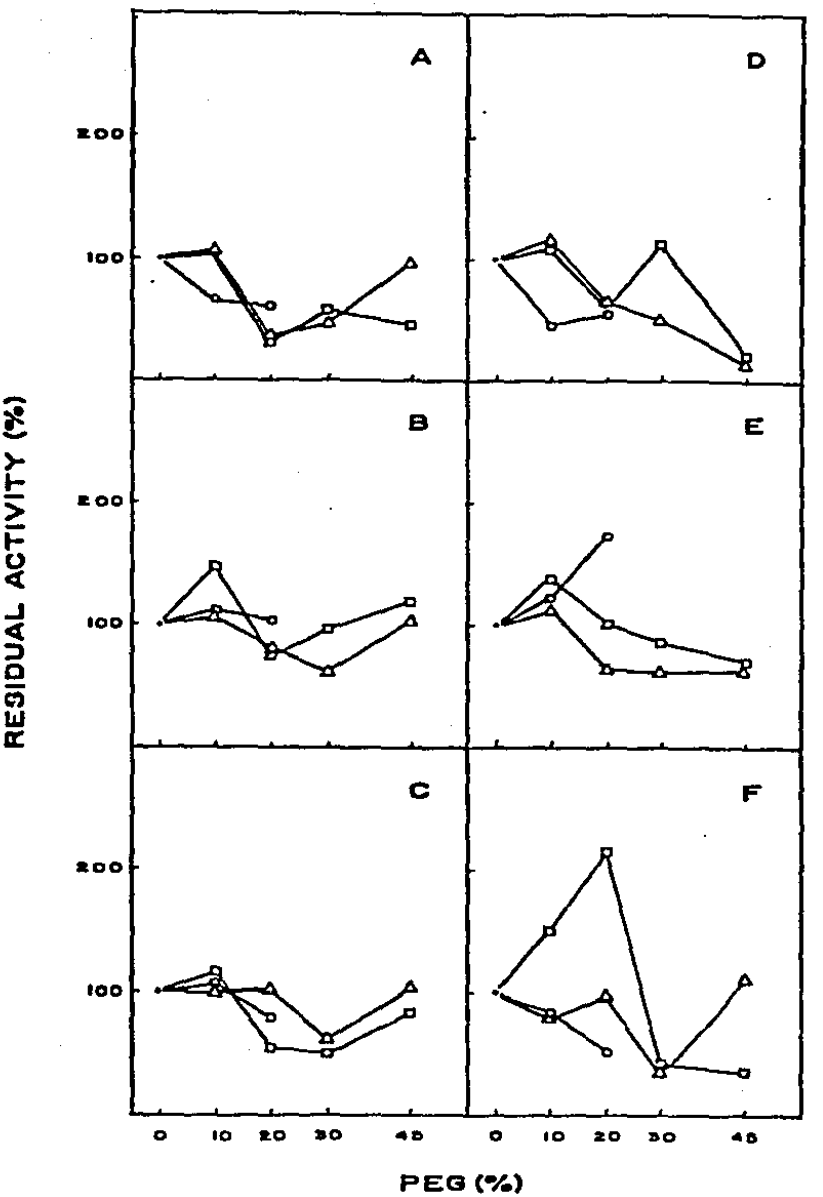

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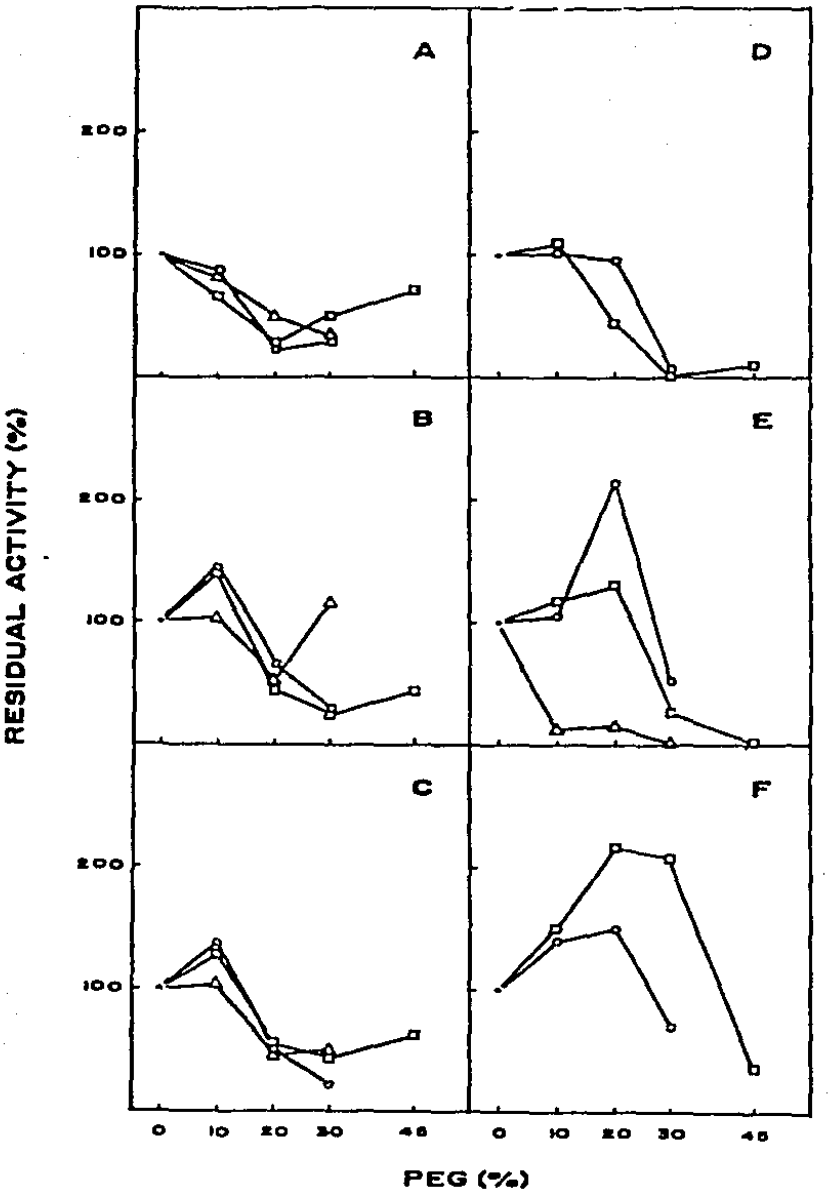

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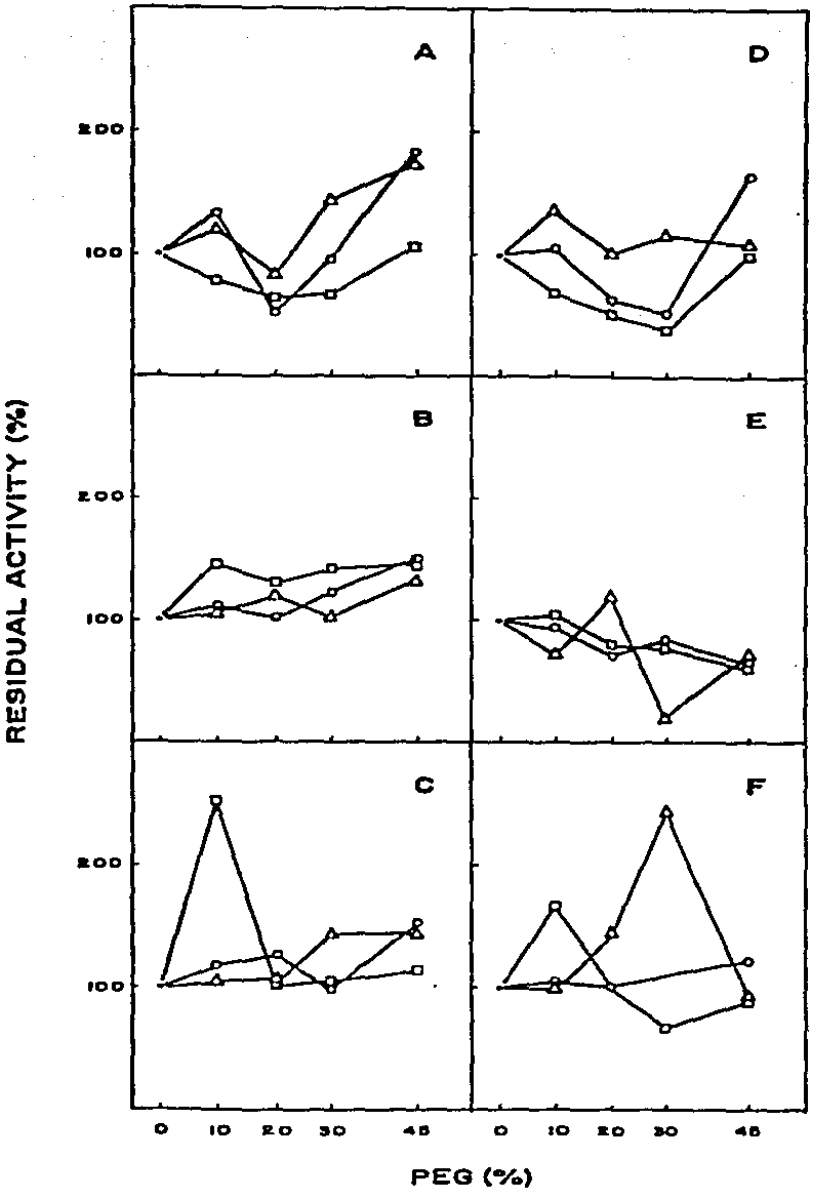

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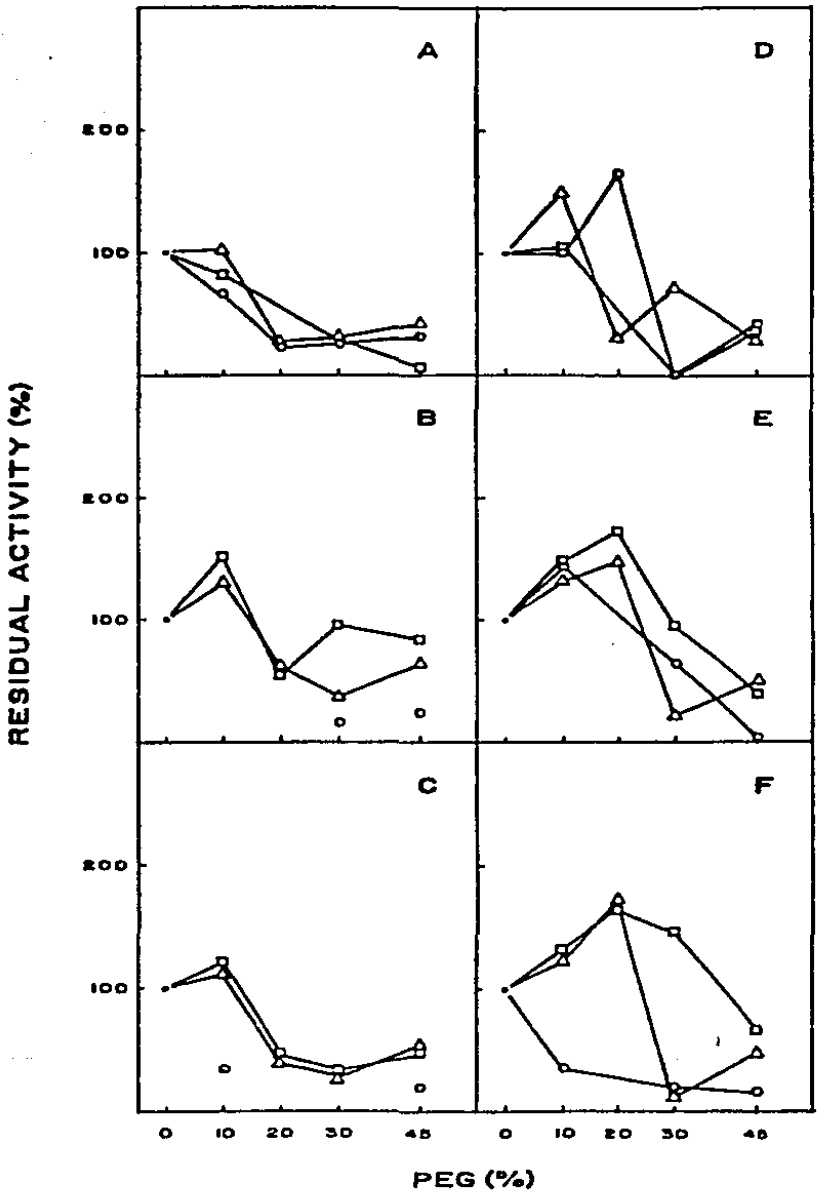

Figure 8.

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In vitro effects of PEG and proline on ammonium assimilation enzymes of maize

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Summary

In order to determine which could be the pathway of ammonium assimilation during stress, the effects of polyethyleneglycol and proline in glutamine synthetase and glutamate dehydrogenase activities were studied employing the *in vitro* system of Paleg *et al* (Plant Physiol. 75: 974-978, 1984). A differential action of polyethyleneglycol on the enzymes extracted from leaves and roots was observed. The results suggest that in the roots ammonium assimilation could be accomplished by glutamate dehydrogenase since it proved to be more resistant to osmotic stress and the affinity of the stressed enzyme for ammonium was increased 100% by proline.

Key words: *Zea mays* L., glutamate dehydrogenase, glutamine synthetase, *in vitro* stress.

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase; PEG, polyethyleneglycol; PVP, polyvinylpyrrolidone.

Introduction.

The accumulation of free amino acids, and particularly proline, in plants grown under water stress has been well documented (Aspinall and Paleg 1981, Shevyakova 1983). It has been proposed that proline accumulation is a mechanism of osmoregulation. However, there is little evidence to support this explanation. One conceptual approach in the search for a role for proline has centered on the amelioration of deleterious effects of heat, pH, salts, chemicals and drought on enzyme activity in in vitro cell and organelle systems (Pollard and Wyn Jones 1979, Paleg et Al 1981, Ahmad et Al 1982, Nash et Al 1982, Paleg et Al 1984). In all reported cases, proline provided a concentration-dependent protection against a range of stress conditions.

On the other hand, high concentrations of proline have been attributed to the inhibition of its oxidation under water stress (Stewart et al 1977), to inhibition of proline incorporation into a proline-rich protein (Shiralipour and West 1984a), and to the continuous synthesis of this amino acid under stress conditions (Shiralipour and West 1984b). In the latter, the incorporation of nitrogen, nitrate or ammonium for amino acid synthesis would be necessary and either GS and GDH, or both, must be active for such function. It has been reported that under in vitro osmotic stress GS activity is lowered (Paleg et Al 1984) and GDH activity increased (Becana et Al 1984, Kaur et al 1985). Nevertheless, there is no conclusive evidence to show which enzyme remains active during water stress.

The present study describes the interactions between GS, GDH, PEG-6000 and proline in the *in vitro* system used by Paleg and coworkers (Paleg *et al* 1984) in order to determine which enzyme could be responsible for the incorporation of ammonia into amino acids.

Materials and methods

Plant material. Maize plants (*Zea mays* L. var. Chalqueño criollo) were grown in moist agrolite and watered daily for 10 days before harvesting. They were kept in a growth chamber at 25°C with a 12 h photoperiod at 3000 lux.

Enzyme extraction. Tissues were frozen with liquid nitrogen immediately after harvesting and ground to a fine powder with mortar and pestle. The powder was then homogenized in a Polytron for 2 min with 2.5 volumes (w/v) of extraction buffer (50 mM Tris-HCl pH 8.2, 1 mM $CaCl_2$, 5 mM mercaptoethanol and 5% PVP). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 14,000 x g for 30 min. All manipulations were done at 4°C. The supernatant was employed for PEG-induced precipitation.

PEG-induced precipitation. Precipitation was carried out in a volume of 1.0 ml PEG (PEG-6000, J.T. Baker) and/or proline were dissolved in 0.4 ml 50 mM Tris-HCl, pH 6.5, prior to the addition of the crude extract. After adding the extract, the samples were vortexed and allowed to stand in ice for at least 45 min. The samples were centrifuged at 3,000 x g for 15 min and the

supernatants used for enzyme activity determinations.

Enzyme assays. NAD-, NADH-GDH and GS assays were performed as described by Loyola-Vargas and Sánchez de Jiménez (1984, 1986). Aminative and deaminative GDH activities were defined as nmoles of cofactor reduced or oxidized per minute, respectively, and GS activity was expressed as μmoles of γ-glutamyl-γ-hydroxamate formed per min. Protein content was determined according to Peterson (1977).

Results

GS activity from the root diminished when PEG was present; at 30% PEG only 20% of GS activity remained in solution. However, with 1 M proline present, 60% of the original activity remained with 80% PEG (Figure 1). These results contrast markedly with those obtained with NAD(H)-GDH from the root. GDH remained in solution at high concentrations of PEG independently of the presence of proline (Figure 2).

The response obtained with the enzymes extracted from the leaves was completely different to that obtained for the root. In the presence of PEG, first leaf GS activity remained the same whether or not there was 1 M proline in the system (Figure 3). We observed a similar response with second leaf GS (data not shown). Even though NAD(H)-GDH patterns of activity in the first (data not shown) and second leaves (Figure 4) in the presence of PEG were independent of proline concentration, only 20% of the

activities remained in solution, in contrast to the 70% found in the GDH from the root.

Ammonium Km values for root GDH were not modified by 50% PEG or by 1 M proline. However the enzyme showed an increment of 100% in its affinity for ammonium when PEG and proline were present simultaneously (Table 1).

Discussion

The results show that relatively high concentrations of proline prevent the precipitating action of PEG; proline action is highly selective since it greatly affects GS activity from the root but not from the leaves. However PEG stress has no effect on aminative or deaminative root GDH activities. These results suggest that *in vivo* stress may exert a differential action on different enzymes, in this particular case, on the two enzymes responsible for ammonium assimilation in higher plants (Miflin and Lea 1980). There is still a lot of controversy on how plants subjected to different types of stress assimilate nitrogen. In some systems it is suggested that GDH could be the key enzyme (Kaur *et al* 1985), but in others, the GS/GOGAT cycle is considered to be more important (Bottacin *et al* 1985). Our results show clearly that GS activity in roots is sensitive to *in vitro* PEG effects, while GDH is more resistant. This may suggest that, in maize roots, ammonium assimilation during stress is accomplished by GDH since it not only remains in solution but also its affinity for this ion is increased 100%. This may be due

to a conformational change in the enzyme as a result of thermodynamic interactions of the system solute-water with proteins; proline may bind water to proteins and thus maintain their hydration. High proline levels (35 μmol g dry wt^{-1}) are accumulated in maize plants as a result of drought, as Ibarra and Sánchez de Jiménez (personal communication) and Ilahi and Dorffling (1982) have observed *in vivo*. In the leaves, the alternative situation may occur and the GS/GOGAT cycle could be functioning during stress *in vitro*. On the other hand, it is known that there are two GS, cytoplasmic and chloroplastic, and that their ratio varies according to the species. In maize leaves, both GS occur in approximately the same quantities (McNally *et al* 1983) and cytoplasmic GS of photosynthetic tissues is very similar to GS of non-photosynthetic tissues. The results shown in figure 3 suggest that the cytoplasmic GS isoenzyme is sensitive to stress which would agree with GS behaviour in the roots. In summary, PEG exerts an inactivating effect on the ammonia assimilating enzymes in maize. The response to PEG depends significantly on the tissue from which these enzymes are extracted and PEG not only causes enzyme inactivation but also changes in their kinetic parameters.

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Figure legends

Figure 1. Residual root GS activity after PEG treatment in the presence (□), and absence (●), of 1 M proline.

Figure 2. Residual NADH- (A) and NAD-GDH (B) activities from the roots after incubation with PEG (●), and PEG plus 1 M proline (□).

Figure 3. Residual GS activity from the first leaf after PEG treatment with (□), and without (●), 1 M proline.

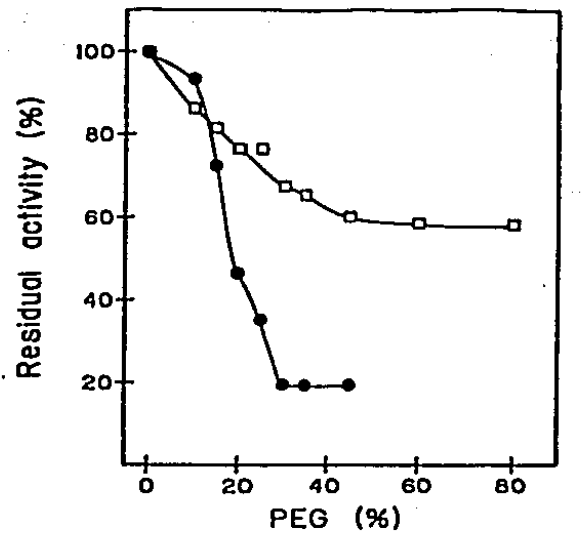
Figure 4. Residual NADH- (A) and NAD-GDH (B) activities from the second leaf after incubation with PEG (●), and PEG plus 1 M proline (□).

TABLE 1

Ammonium Km values for GDH under different conditions

TREATMENT	NH_4^+ - Km (mM)*	
	ROOT	FIRST LEAF
NONE	28.8	23.3
+50% PEG	28.8	16.5
+1 M proline	23.5	16.1
+50% PEG +1 M proline	13.7	31.7

* For Km determinations, PEG and/or proline was added to the reaction mixture. After PEG and/or proline treatment the supernatant phase was used as the enzyme source. Km values were calculated from Lineweaver-Burk plots.



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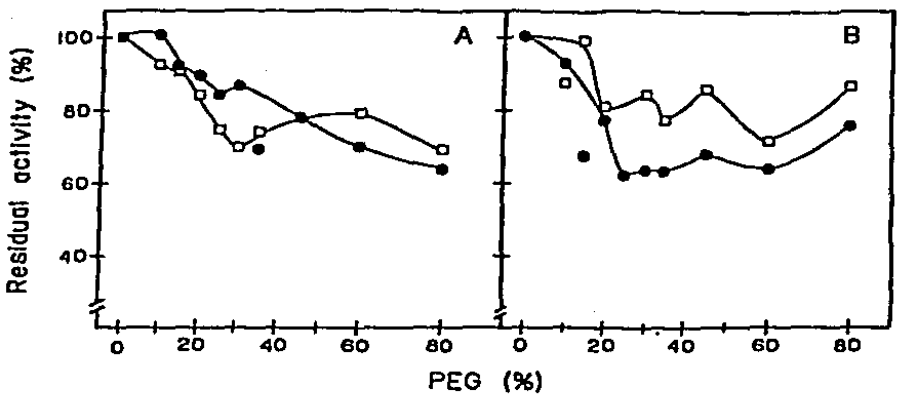

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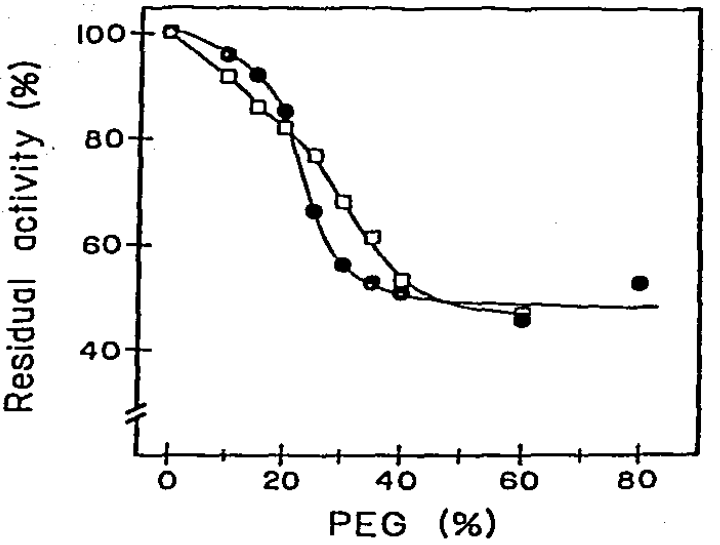

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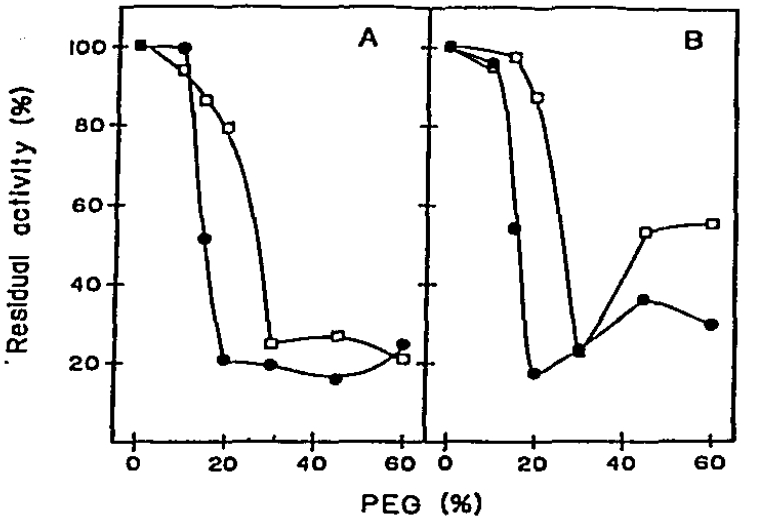

Figure 4.