Plant Molecular Biology Reporter 11(3) 1993 220–229

Protocol

Efficient and Sensitive Assay for T-DNA-Dependent Transient Gene Expression

L. Rossi, J. Escudero, B. Hohn and B. Tinland

Friedrich Miescher-Institut, P.O. Box 2543, CH4002 Basel, Switzerland.

Key Words : β-glucuronidase, transient expression, T-DNA, tobacco seedlings, *Nicotiana tabacum*

Abstract: We describe here a very sensitive and reproducible method to detect the efficiency of *Agrobacterium*-mediated T-DNA transfer. This method is based on a quantitative assay of β-glucuronidase activity produced in the plant cell upon transfer of T-DNA carrying a special *uidA* gene construct. Analysis of the transfer efficiency of a transfer-proficient bacterium compared with that of the same bacterium diluted at different ratios with a transfer-defective bacterium shows a high sensitivity of the β-glucuronidase activity in the plant. Five orders of magnitude in T-DNA transfer efficiency can be covered when the activity is measured combining the fluorimetric MUG assay (for high activity) and the histochemical X-Gluc assay (very sensitive for low activity).

Agrobacterium tumefaciens is a plant pathogen that can induce tumors on dicotyledonous plants. The development of the tumor is caused by the expression of oncogenes, which are transmitted from the bacterium to the plant cell on a well-defined piece of DNA called transferred DNA (T-DNA). The T-DNA is delimited by two imperfect 25-bp direct repeats, the right and left borders, and is located on the Ti-plasmid (tumor-inducing plasmid). The Ti-plasmid also contains the so-called *virulence region,* which encodes the proteins (Vir

---

Abbreviations: T-DNA, transferred DNA; MUG, 4-methyl-umbelliferyl-glucuronide; Ti-plasmid, tumor-inducing plasmid; X-Gluc, 5-bromo-4-chloro-3-indolyl-glucuronide; *uidA*, gene encoding β-glucuronidase; GUS, β-glucuronidase. Other abbreviations are defined under *Materials, Solutions.*

proteins) necessary for the T-DNA transfer process. The practical interest in *Agrobacterium* as a vector for plant transformation and the interest in the mechanism of T-DNA transfer itself has led to a series of studies aimed at defining and characterizing the steps of *Agrobacterium*-mediated plant transformation (for reviews see Ream, 1989; Kado, 1991; Zambryski, 1992; Hooykaas and Schilperoort, 1992). Determination and quantification of the transfer was done using transient assay systems in which the expression of the marker genes does not require stable integration in the plant genome (histochemical β-glucuronidase staining (Jansen and Gardner, 1989), agroinfection (Grimsley et al., 1986)).

We describe here a transient assay in which we allow *A. tumefaciens* to transfer the T-DNA carrying an improved *uidA* gene to the cells of young tobacco seedlings. We precisely quantify the amount of β-glucuronidase activity produced in the plant cell using fluorimetric (MUG) and histochemical (X-Gluc) enzyme assays (Jefferson, 1987). These assays represent the most sensitive method to quantify the T-DNA transfer to plant cells and can be used for a wide range of purposes.

Materials

Solutions

MS medium: (Murashige and Skoog, 1962)

YEB: (Sambrook et al., 1989)

The following solutions are described by Jefferson (1987):

GUS extraction buffer: 50 mM sodium phosphate buffer, pH 7.0; 10 mM DTT; 1 mM Na_2EDTA; 0.1% sodium lauryl sarcosinate; 0.1% Triton X-100

MUG solution: 1 mM MUG in GUS extraction buffer

MUG stop solution: 0.2 M Na_2CO_3

GUS staining buffer: 100 mM sodium phosphate buffer pH 7.0 with 0.05% X-Gluc dissolved in dimethyl formamide, in the presence of 0.1% sodium azide

Construction of the transfer-defective *A. tumefaciens* strain. Strain GV3101(pPM6000) (a cured C58 nopaline strain containing the pTiAch5 derivative pPM6000, which is deleted in the T-DNA) (Bonnard et al., 1989), was modified in the *virD2* gene of the Ti-plasmid, yielding strain GV3101(pPM6000K). This latter strain lacks 70% of the coding sequence of the VirD2 protein, which is essential for T-DNA transfer. This strain was shown to be T-DNA transfer-defective (Rossi et al., 1993). The transfer-proficient strain GV3101(pPM6000) contained an intact *virD2* gene.

Description of the *uidA* gene construct.
We used the binary vector pLRG (Rossi et al., 1993) containing an improved *uidA* gene from pGUS23 (Schultze et al., 1990; Puchta and Hohn, 1991) in the T-DNA.

The *uidA* gene is under control of the 35S promoter and the translational start site is derived from gene V of cauliflower mosaic virus (Schultze et al., 1990).

Procedures

Cocultivation of tobacco seedlings with *A. tumefaciens* (see Fig. 1)

- 3 mL fresh overnight culture of *A. tumefaciens* grown in YEB medium containing the antibiotics rifampicin (100 mg/mL) and gentamycin (40 mg/mL) was washed twice with 3 mL 10 mM $MgSO_4$ and resuspended in 10 mL MS medium to a final A_{600} = 0.6.
- 100 *Nicotiana tabacum* SR1 plantlets were added to the bacterial suspension. The plantlets had been grown for one to two weeks on sterile, wet Whatman paper in a growth chamber at 25°C with 16 h light/day.
- The mixture was exposed to reduced pressure (0.15 atm) in a sterile vacuum chamber for 5 minutes.
- The seedlings were placed on MS plates (1% agar) and further cocultivated for three days in a growth chamber (25°C 16 h light/day).
- The plantlets were washed in sterile 10 mM $MgSO_4$ and blotted dry on sterile Whatman paper.
- Part of the plantlets of each single cocultivation was analyzed with the fluorimetric MUG assay, another part with the histochemical GUS assay.

Fluorimetric MUG assay.

- 30 plantlets were added to 400 mL of GUS extraction buffer and homogenized in a 1.5-mL Eppendorf tube.
- The mixture was centrifuged for 10 minutes at 18,000 *g* at 4°C. The supernatant was considered as protein extract.
- The protein concentration of the extract was determined according to Bradford (1976).
- 10 µL of the extract was added to 200 µL of MUG solution in the first row of a multiwell plate (Dynatec Microfluor).
- Immediately afterwards, 20 µL of the reaction mixture in the first row was transferred to 200 µL of stop solution added in the second row. This became the zero time-point for the enzymatic assay.
- The plate was incubated at 37°C.

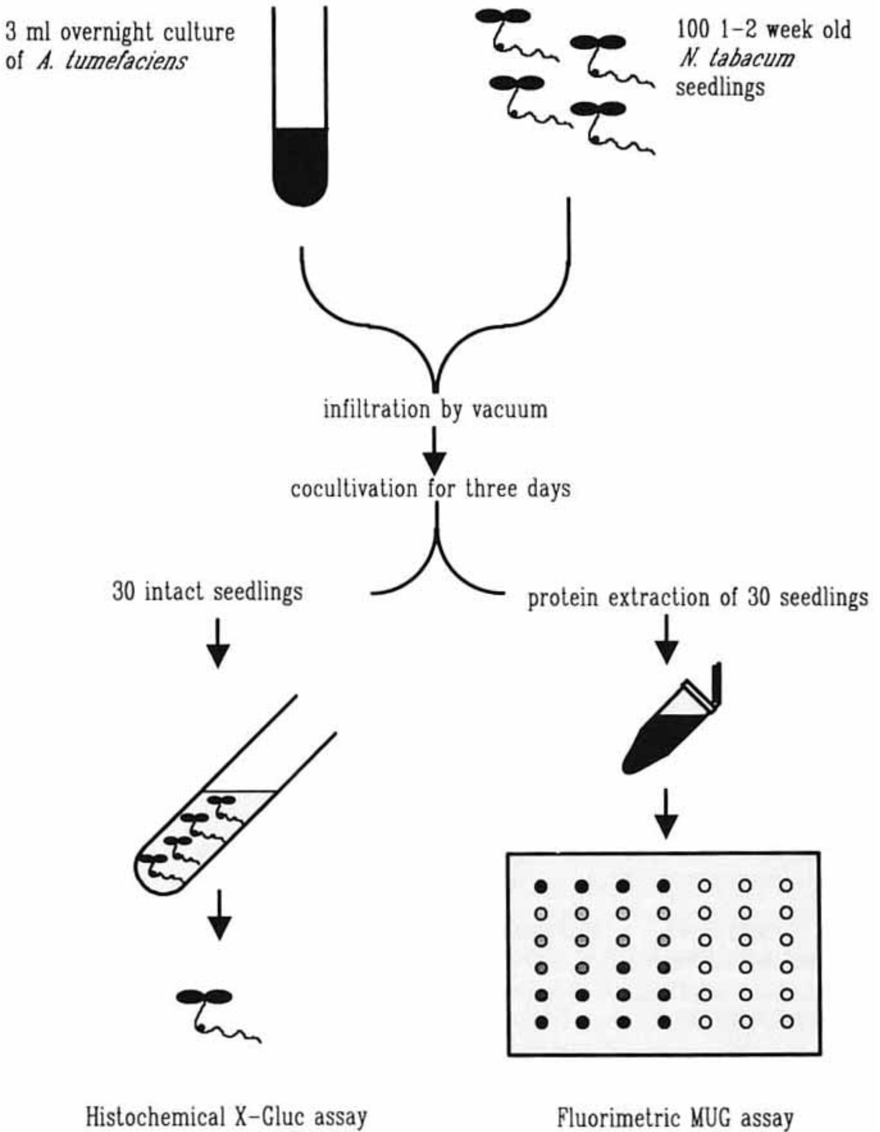


Fig. 1. Schematic representation of the experimental set-up.

- Subsequently every hour 20 µL of the reaction mixture in the first row was transferred to 200 µL of stop solution in the subsequent rows.
- The fluorimetric signal of each sample was determined with a Titertek Fluoroskan II fluorimeter (excitation at 365 nm, emission at 455 nm).
- From the initial slope of the curve obtained by plotting the fluorimetric value against the time, we calculated the value of the enzymatic activity in each sample.
- These values were normalized to the protein concentration of each extract and the relative values (compared to the wild-type) of enzymatic activity of the different extracts were determined.

Histochemical X-Gluc assay.

- After cocultivation, 30 plantlets were added to 5 mL of GUS staining solution and soft vacuum (0.15 atm) was applied for 5 minutes in order to standardize the distribution of the substrate into the cells of different tissues in the plantlets. The reaction was allowed to proceed at 37°C for 24 hours.
- The plantlets were washed in sterile water and bleached with ethanol.
- The blue spots present on the plantlets were counted under the binocular.

Table I. Detection of β–glucuronidase activity upon T-DNA transfer to tobacco cells. The transfer-proficient *Agrobacterium* strain was diluted in different ratios with a transfer-defective strain.

Dilution proficient/defective	Enzymatic Activity normalized[1]	Blue spots No.[2]	Blue spots normalized[3]
proficient strain	1000	2100	1000
10^{-1}	120 ± 43	1530	730
10^{-2}	14 ± 4.0	631	300
10^{-3}	1.7 ±0.25	267	130
10^{-4}	0.64 ±0.20	20	10
10^{-5}	0.52 ±0.10	1	0.5
defective strain	0.37 ±0.025	0	0

[1]MUG assay; activity of proficient strain normalized to 1000.
[2]X-Gluc assay; number of blue spots in 30 plantlets.
[3]Number of spots of proficient strain normalized to 1000.

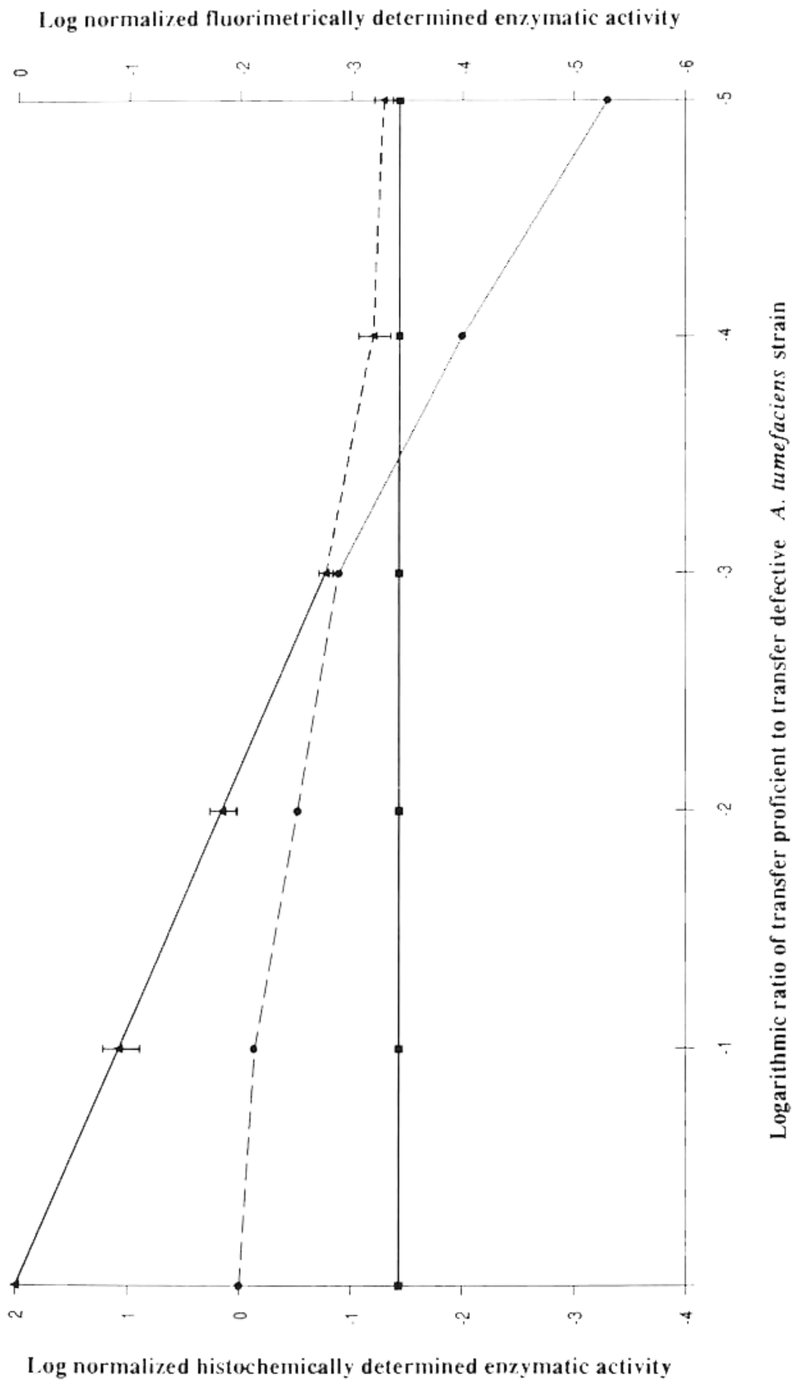
Results

In order to assess the sensitivity of the assay, the transfer-proficient *A. tumefaciens* strain GV3101(pPM6000, pLRG) was diluted in different ratios with the transfer-defective strain GV3101(pPM6000K, pLRG) and the transfer efficiency was measured with the MUG assay (see Procedures). In Fig. 2 the results obtained in three separate experiments are plotted on a logarithmic scale. We can observe a difference in the β-glucuronidase activity of three orders of magnitude between the transfer-proficient strain and a 1-to-1000 dilution of this strain. Furthermore, even the value for the dilution 10^{-3} is clearly above the background (see Table I), whereas further dilutions showed a non-linear response, probably indicating the limit of detection of the assay. The values of the standard deviation given in Table I and represented in Fig. 2 are very low. Thus, the values of the normalized enzymatic activity measured with the MUG assay can be considered reliable and precise.

In parallel with the MUG assay we performed the histochemical X-Gluc assay. In Table I we present the number of blue spots counted in one experiment after staining 30 plants per dilution. We found that an accurate counting of the number of spots was very difficult when the plantlets exhibited a large number of blue spots throughout the whole plant tissue. Spots of different color intensity could also be found. A comparison of the results obtained by the MUG assay with those obtained by the histochemical assay indicates that the histochemical assay underestimates the number of T-DNA transfer events when high concentrations of the transfer-proficient bacterial strain were involved. At low concentrations of the transfer-proficient bacteria (dilutions $1{:}10^3$ to $1{:}10^5$), where the number of blue spots can be determined more precisely, the slope of the plot of the histochemical X-Gluc assay is similar to the slope of the curve representing the enzymatic MUG assay in the region that is considered to be reliable. Thus the histochemical staining seems to be very adequate to detect rare T-DNA transfer events. For this reason the Y-axis for the two measurement in Fig. 2 is shifted by two orders of magnitude.

Discussion

In the assay described here we measured the enzymatic activity of β-glucuronidase, an enzyme produced upon transfer of T-DNA containing the gene for it. The assay is sensitive, with a low background activity; reproducible; and quantitative over a wide range of concentrations.



Log normalized fluorimetrically determined enzymatic activity
0
-1
-2
-3
-4
-5
-6
Logarithmic ratio of transfer proficient to transfer defective A. tumefaciens strain
-5
-4
-3
-2
-1
2
1
0
-1
-2
-3
-4
Log normalized histochemically determined enzymatic activity

The high *sensitivity* of the assay is most probably due to the improved *uidA* gene construct used. The sequence surrounding the start codon is derived from gene V of CaMV (Schultze et al., 1990). This allows efficient translation, with negligible background activity stemming from *A. tumefaciens*. A similar construct was described earlier (Jansen and Gardner, 1989). The remarkable sensitivity of the assay most probably also reflects the quality and competence of the tobacco seedlings to act as recipient for *Agrobacterium*-mediated DNA transfer.

We attribute the *reproducibility* of the assay to the fact that each measured value stems from a large number of seedlings. Thus a statistically significant sampling is possible, avoiding plant to plant differences. The assay is *quantitative* over a wide range of concentrations of transfer competent *Agrobacterium* cells. This applies to the fluorimetric assay, for which extracts of many seedlings guarantee standardized measurements. The background inherent to the system does not allow monitoring of T-DNA transfer efficiencies comparable to a ten-thousand-fold dilution of our standard strain. The histological staining is not accurate in the higher ranges of concentration (activity), due to many cotransfer events and to confluence of blue spots (the first product of the reaction catalyzed by β-glucuronidase is known to diffuse out from the plant cell of origin (De Block and Debrower, 1992)), but allows for a fairly precise measurement in the lower concentration (activity) ranges due to the non-existent background of a blue spot. A combination of the two methods of detection thus covers almost five orders of magnitude.

Both detection methods most probably assay transient expression of T-DNA not (yet) integrated in the plant genome (Jansen and Gardner, 1989; Machida et al., 1992). Thus the gene activity of the T-DNA units that entered into the plant cell nucleus is measured, irrespective of integration. Therefore problems in the interpretation of the results linked to position effect on gene expression can be avoided. We interpret β-

---

Fig. 2. Logarithmic plot of the normalized β-glucuronidase activity (opposite). Gus activity was measured with the MUG assay (triangles) and the X-Gluc assay (circles). The measured activity represents the efficiency of T-DNA transfer of various concentration of the transfer-proficient *Agrobacterium* strain GV3101(pPM6000, pLRG). Dilutions were done against the transfer defective *Agrobacterium* strain GV3101(pPM6000K, pLRG). The dotted part of the respective curves represents the region of the assays where an accurate measurement is difficult. The background activity (squares) in the MUG assay was measured after cocultivation of the transfer deficient strain GV3101(pPM6000K, pLRG) with tobacco seedlings.

glucuronidase activity as measured by the fluorimetric assay as being strictly dependent on the number of T-DNA units entering a plant cell nucleus. The measured values therefore are likely to be proportional to the number of T-DNA units entering the nuclei (no matter how many nuclei). In contrast, data from the histochemical staining are likely to be proportional to the number of nuclei that have received at least one expressing T-DNA unit.

The described assay can be used to analyze any parameter important for T-DNA transfer, such as analysis of virulence gene activity (Rossi et al., 1993), of *cis* acting sequences or other factors such as virulence gene inducers that contribute to efficiency of T-DNA transfer.

Acknowledgments. We are grateful to S. Kocher for drawing Fig. 1, to R. Beffa and T. Ichikawa for critically reading the manuscript. JE was partially supported by a Fellowship from the Spanish Ministerio de Educación y Ciencia while on leave of absence from Instituto Nacional de Investigaciones Agrarias, Madrid (Spain).

References

Bonnard, G., B. Tinland, F. Paulus, E. Szegedi and L. Otten. 1989. Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Mol. Gen. Genet. 216:428-438

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Bioch. 72:248-254.

De Block, M. and D. Debrower. 1992. In-situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. The Plant J. 2:261-266.

Grimsley, N., B. Hohn, T. Hohn and R. Walden. 1986. "Agroinfection, " an alternative route for viral infection of plants by using the Ti-plasmid. Proc. Natl. Acad. Sci. 83:3282-3286.

Hooykaas, P.J.J., and R.A. Schilperoort. 1992. *Agrobacterium* and plant genetic engineering. Plant Mol. Biol. 19:15-38.

Jansen, B.J. and R.C. Gardner. 1989. Localised transient expression of GUS in leaf discs following cocultivation with *Agrobacterium*. Plant Mol. Biol. 14:61-72.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Reporter 5:387-405.

Kado, C.I. 1991. Molecular mechanisms of crown gall tumorigenesis. Crit. Rev. Plant Sci. 10:1-32.

Machida, Y., N. Shimoda, A. Yamamoto-Toyoda, Y. Takahashi, R. Nishihama, S. Aoki, K. Matsuoka, K. Nakamura, Y. Yoshioka, T. Ohba and R.T. Obata. 1992. Molecular interactions between *Agrobacterium* and plant cells. In: Nester, E.W. (ed) *Advances in Molecular Genetics of Plant-Microbe Interactions*. Vol. 2:85-96. Kluwer, Dordrecht.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15:473-493.

Puchta, H. and B. Hohn. 1991. A transient assay in plant cells reveals a positive correlation between extrachromosomal recombination rates and length of homologous overlap. Nucleic Acids Res. 19:2693-2700.

Ream, W. 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annu. Rev. Phytopathol. 27:583-618.
Rossi, L., B. Hohn and B. Tinland. 1993. The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. Mol. Gen. Genet. 239:345-353.
Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor).
Schultze, M., T. Hohn and J. Jiricny. 1990. The reverse transcriptase gene of CaMV is translated separately from the capsid gene. EMBO J. 9:1177-1185.
Zambryski, P.C. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43:465-490.

Broaden your knowledge of plant mineral nutrition
with this timely journal from Marcel Dekker, Inc....

JOURNAL OF PLANT NUTRITION

Executive Editor:
J. BENTON JONES, JR.
Georgia University Station, Athens

Featuring careful evaluation and selection of the most current, useful research reports as well as review articles, the ***Journal of Plant Nutrition*** provides plant nutritionists with valuable information on the influences of known essential and non-essential mineral elements on plant physiology and growth.

Selected Contents from Volume 16, Number 2, 1993

- **Plant Mineral Nutrition in Precollege Education,** *D. R. Hershey*
- **Differential Responses of Alfalfa Clones to Aluminum-Toxic Acid Soil,** *V. C. Baligar, T. A. Campbell, and R. J. Wright*
- **Calcium ($^{45}Ca^{2+}$) Absorption Inhibition by Aluminum (AL^{3+}) in Sorghum Roots,** *R. E. Wilkinson and R. R. Duncan*
- **Preliminary Evaluation of Experimental Techniques for Studying the Absorption of Rubidium by Grape Roots,** *J. R. Norton and R. C. Smith*
- **Yield of Iron-Sprayed and Non-Sprayed Strawberry Cultivars Grown on High pH Calcareous Soil,** *H. Z. Zaiter, I. Saad, and M. Nimah*
- **Potassium and Sodium Fertilization Affects Leaf Nutrient Content and Growth of 'Shawnee' Blackberry,** *J. M. Spiers*
- **The Effect of Amino Acids and Amides on the Regulation of Nitrate Uptake by Wheat Seedlings,** *C. O. Rodgers and A. J. Barneix*

Refereed by an internationally renowned editorial board, the ***Journal of Plant Nutrition*** is an excellent resource for horticulturists, botanists, soil scientists, agronomists, plant physiologists, and phytologists.

1993 Volume 16, 12 Numbers
Institutional Rate: $695.00
Individual Rate: $347.50

▸ For a complimentary copy of the *Journal of Plant Nutrition*,
or for subscription information, contact the **Promotion Department** at:

MARCEL DEKKER, INC.
270 Madison Avenue
New York, NY 10016
Tel. (212) 696-9000 FAX: (212) 685-4540