

Transformation of tropical grain legumes using particle bombardment

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The particle bombardment method for direct gene transfer was used to introduce β -glucuronidase (GUS) and neomycin phosphotransferase (NPTII) genes into three *Vigna* species. Relatively high frequency of transient GUS expression was observed on all parts of germinating embryos 18 to 24 hours after bombardment. Factors such as the ratio of gold particles to plasmid DNA carrying the genes of interest, pressure used for delivery of the particles into tissues, and the number of times the tissues were bombarded all affected the efficiency of transient GUS expression. In addition, incubation of tissues on kinetin enhanced the expression of GUS. These results indicate that the particle delivery system could effectively be used to introduce genes into meristematic regions of *Vignas*.

The genetic modification of crop plants has undergone a revolution in the past few years due to the development of new *in vitro* culture systems and novel transformation techniques. Transformation, however, continues to be limited by such factors as the recalcitrant nature of some plant species to regenerate and by the host range specificity of *Agrobacterium* strains most often used as vectors for introducing new genes into plant cells.

Vigna aconitifolia (Jacq.) Marchal (moth bean), *V. radiata* L. Wilczek (green gram) and *V. mungo* (black gram) are important tropical grain legumes. *V. aconitifolia* has been found to be highly amenable to regeneration whereas *V. radiata* and *V. mungo* have proven to be recalcitrant¹. Transformation of *V. aconitifolia* protoplasts either by direct DNA uptake^{2,3} or cocultivation with *A. tumefaciens*⁴, yielded kanamycin resistant callus tissues but no plantlets were regenerated. The frequencies of transformation were found to vary significantly depending on the plant cultivar. Transformation of *V. radiata* has been reported recently⁵. Cocultivation of cotyledons with *A. tumefaciens* yielded transformed tissues, but it is not clear whether transgenic plants were regenerated.

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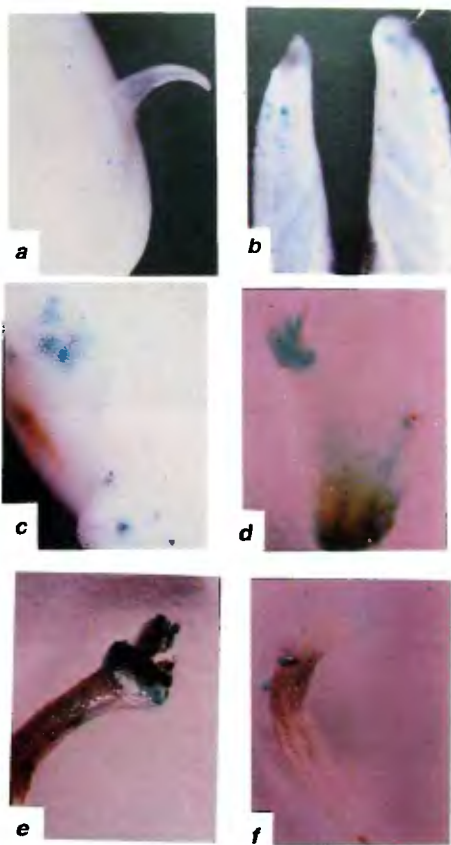


Figure 1 a-f. Expression of GUS activity on various parts of germinating mature bean embryos from *V. radiata* (a e) and *V. mungo* (f). a, Mature embryo attached to cotyledon ($\times 12.5$), b, Primary leaves ($\times 32$), c, Cotyledon ($\times 32$), d, Cotyledonary node region and hypocotyl base ($\times 12.5$), e, Epicotyl and cut apex (primary leaves removed) ($\times 6.3$), f, Cotyledonary node region ($\times 12.5$).

In this report, we demonstrate particle bombardment-mediated gene transfer to ungerminated mature embryos of *V. aconitifolia* (cv. RMO-40; TCVA-1), *V. radiata* (cv. ML-5; K-851) and *V. mungo* (cv. T-9; RU-19) of a reporter and a selectable marker gene, β -glucuronidase (GUS) and neomycin phosphotransferase (NPTII), respectively. Particle bombardment-mediated gene transfer has the ability to deliver genes directly into any



Figure 2. Expression of GUS on various parts of six-day seedling of *V. radiata* ($\times 6.3$)

Table 1. Average number of GUS expression units^a on *Vigna* embryos 18 hours after particle bombardment

Pressure of bombardment (p s i) ^b	<i>V. aconitifolia</i>	<i>V. radiata</i>	<i>V. mungo</i>
900	20	50	0
1300	106	115	21
1550	114	181	50
1800	168	198	85

^a An expression unit represents a blue spot, whether an aggregate of cells or a single cell

^b pounds per square inch

cell, tissue or organelle⁶. The technique relies upon bombardment of recipient cells with high-velocity tungsten or gold microprojectiles which are coated with foreign DNA.

Mature ungerminated embryos were obtained from seeds soaked overnight in sterilized distilled water. After removal of the seed coat and one of the cotyledons, the embryo attached to the remaining cotyledon was plated on MS (ref. 7) medium with inorganic salts and B5 (ref. 8) organics (MS + B5) for particle bombardment. Gold particles used for tissue bombardment were coated with plasmid pBI221 DNA carrying GUS and NPTII reporter and selectable marker genes, respectively⁹. The DNA precipitation mixture contained 1.5 mg of gold particles (1.0 μ m in diameter) suspended in 25 μ l sterile water, 1.5 μ g plasmid DNA, 50 μ l of 2.5 M CaCl_2 and 20 μ l of 0.1 M spermidine. The components were added in the above order,

vortexed at high speed for 3 minutes at 4°C, and centrifuged for a few seconds at 10,000 rpm to remove the supernatant. Coated particles were then washed with 250 μ l anhydrous ethanol by briefly sonicating, vortexing and repeating the centrifugation to remove the supernatant. Particles were resuspended in 100 μ l of anhydrous ethanol and 10 μ l aliquots were used to bombard embryos with a helium-driven gene gun (Biolistic PDS-1000, DuPont). Embryos were bombarded at a distance of 10 cm from the end of the barrel of the particle gun. Tissues were incubated at 25°C in a photoperiod growth chamber (16 h day, 8 h light) and at various intervals analysed histochemically for transient GUS activity using X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide)¹⁰.

Significant GUS expression in the form of single blue spots or clusters on various parts of germinating *Vigna* embryos is shown in Figure 1. Within 18 hours of bombardment, transient GUS expression was detected with all species of *Vigna*. This expression could be significantly enhanced by incubation of bombarded embryos on medium supplemented with 0.1 μ g kinetin per ml (data not shown). GUS activity was observed in the cotyledonary meristematic region (Figures 1d and f) indicating that regeneration of chimeric shoots composed of transformed and normal cells is possible. This is a significant finding in that *V. radiata* and *V. mungo* have only been regenerated from tissue-cultured meristematic regions¹¹⁻¹⁴. Analysis of GUS expression in germinating seedlings 48 hours after particle delivery produced blue colour visible to the naked eye and 6 days

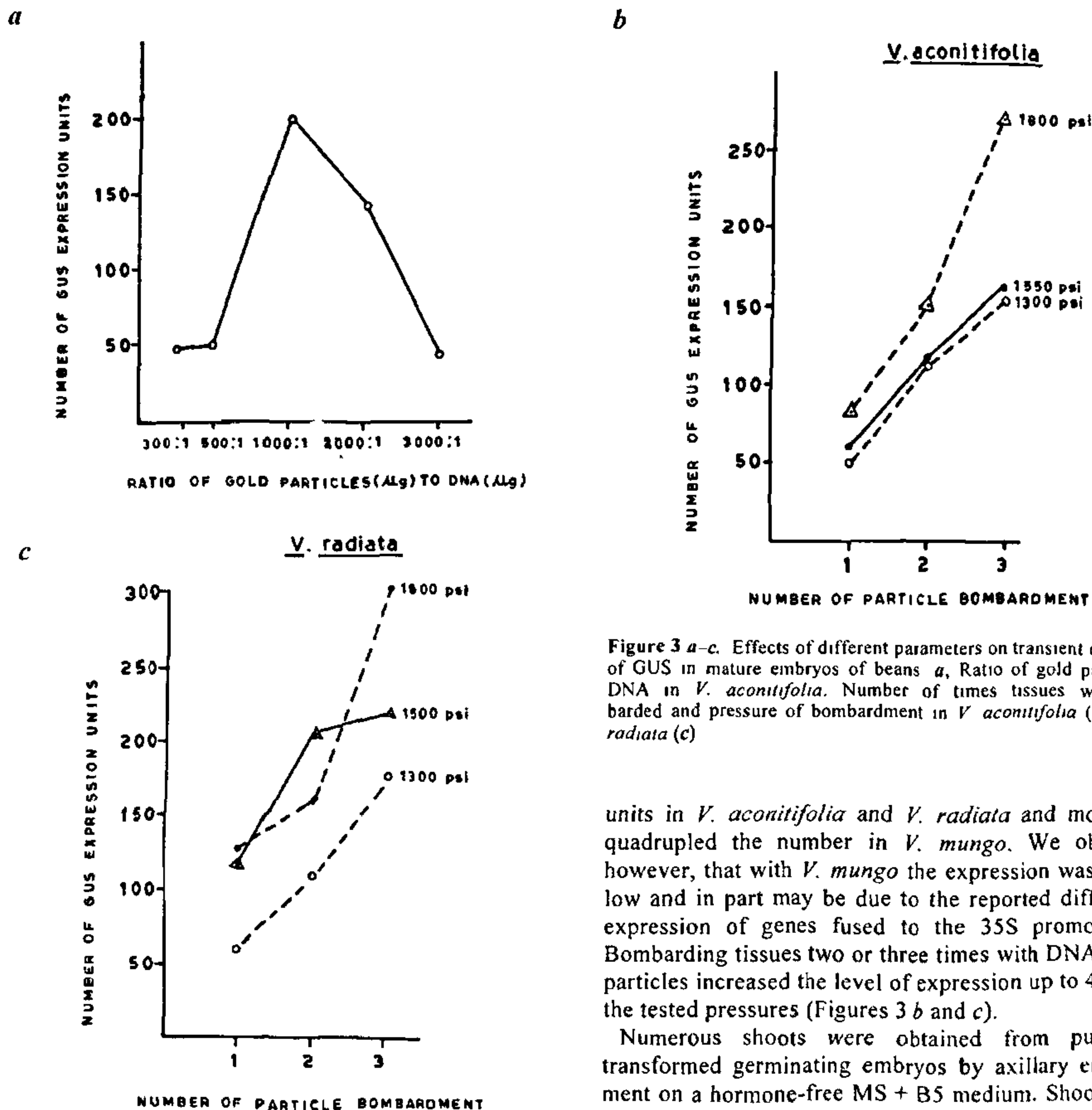


Figure 3 a-c. Effects of different parameters on transient expression of GUS in mature embryos of beans a, Ratio of gold particles to DNA in *V. aconitifolia*. Number of times tissues were bombarded and pressure of bombardment in *V. aconitifolia* (b) and *V. radiata* (c)

units in *V. aconitifolia* and *V. radiata* and more than quadrupled the number in *V. mungo*. We observed, however, that with *V. mungo* the expression was always low and in part may be due to the reported differential expression of genes fused to the 35S promoter^{15, 16}. Bombarding tissues two or three times with DNA-coated particles increased the level of expression up to 4-fold at the tested pressures (Figures 3 b and c).

Numerous shoots were obtained from putatively transformed germinating embryos by axillary enhancement on a hormone-free MS + B5 medium. Shoots were then transferred to MS + B5 medium containing kanamycin at 50 µg/ml to test for expression of the NPTII selectable marker gene. We determined that even though control *V. radiata* and *V. mungo* tissues and shoots could tolerate fairly high levels of kanamycin (400-500 µg/ml), root initiation in apical and axillary shoots was completely inhibited in the presence of 40 µg kanamycin per ml. Since many of the regenerated shoots rooted in the presence of 50 µg kanamycin per ml, they appear to be stably transformed. In addition, rooted plantlets were shown to be GUS-positive using a non-destructive GUS assay¹⁷. Some plantlets reached maturity.

We demonstrate that foreign genes were introduced into mature embryos of various *Ligna* species and cultivars by particle bombardment. The observed expression of the GUS gene in meristematic zones and

later the seedlings were blue all over (Figure 2). No GUS expression was ever observed in tissues bombarded with gold particles not coated with DNA.

The ratio of gold particles to DNA, helium pressure used during bombardment, and the number of times the tissues were bombarded significantly affected the frequency of gene transfer as measured by the GUS assay. GUS expression was maximum at a ratio of 1000 : 1 gold particles to DNA (Figure 3 a). The number of GUS expression units was approximately 4-fold higher at this ratio than at 300 : 1, 500 : 1, or 3000 : 1. A significant increase in GUS expression was observed with increasing pressure associated with the velocity of particles (Table 1) as well as number of bombardments (Figures 3 b and c). Increasing pressure from 1300 to 1800 psi almost doubled the number of GUS expression

rooting of shoots on kanamycin-containing media, indicated that stably transformed plantlets were regenerated. In addition, since regeneration from these zones occurs with minimal hormonal manipulations, the possibility of regenerating somaclonal variants is also minimized.

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Detection and role of chlorotic toxin and phytohormones in the pathogenesis of *Alternaria* blight in *Brassica napus*

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Destruxins are known to play an important role in the pathogenesis of the black spot disease of several *Brassica* species caused by *Alternaria brassicae*. Vivotoxicity of destruxin B was shown by the isolation and characterization from the infected leaves of *Brassica napus*. HPLC purified compound showed chlorotic activity during foliar bioassay on detached *B. napus* leaves. FTIR spectra showed depsipeptide nature of this toxic compound. An

interesting compound antagonistic to the activity of chlorotic toxin was also observed which was removed during purification. This compound was suspected to be a phytohormone due to its ability to induce rooting at the surface of the *B. napus* leaves during foliar bioassay.

DESTRUXINS, a group of cyclodepsipeptides are known to play an important part in the pathogenesis of the entomopathogenic fungus *Oospora destructor*¹ and plant pathogenic fungus *Alternaria brassicae* causing black spot disease on several *Brassica* species². Destruxin B isolated from *A. brassicae* was shown to cause similar symptoms on the leaves of *Brassica* sp. as that of the pathogen. The use of reversed-phase HPLC for preparative isolation of the two major toxins, destruxin B³ and homodestruxin B⁴ from the culture broth of *A. brassicae* was demonstrated by Bains and Tewari² and Tyagi⁵. Buchwaldt and Jensen⁶ reported destruxin B in the leaves of *B. napus* infected by *A. brassicae*. In the present study an attempt was made to look for the presence of destruxin B like compounds in naturally infected leaves of *B. napus*.

Naturally infected leaves (500 g) of *B. napus* cv. BNCN-3 were used to purify the toxin using the method of Tyagi⁵. The leaves were macerated in distilled water (2 l) and filtered through 4 layers of muslin cloth. The filtrate after centrifugation at 5990 g for 15 min was extracted with equal volume of ethyl acetate and flash-evaporated to dryness at 45°C. Dried sample was

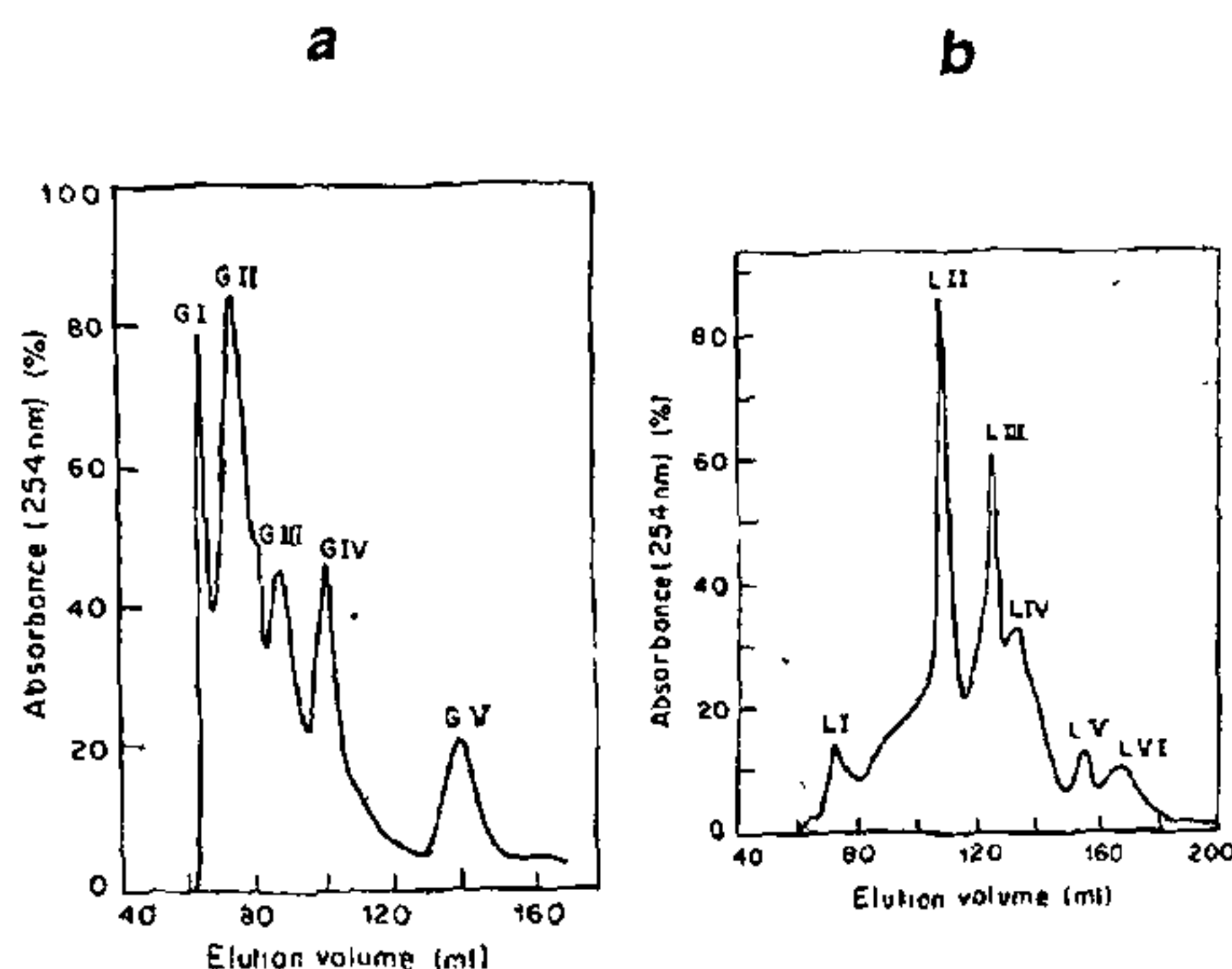


Figure 1. a, Chromatography of crude extract from *Alternaria* blight affected leaves of *Brassica napus* on Sephadex G-10 column (80 × 16 cm), b, Rechromatography of fraction G II from 1 a on Sephadex LH-20 column (80 × 16 cm). L II ~ Chlorotic activity. L III ~ Root induction activity.