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INTERSPECIFIC HYBRIDISATION OF SOYBEANS AND PERENNIAL *GLYCINE* SPECIES INDIGENOUS TO AUSTRALIA VIA EMBRYO CULTURE

P. BROUÉ†, J. DOUGLASS, J. P. GRACE and D. R. MARSHALL

Division of Plant Industry, CSIRO, Canberra City, A.C.T., Australia 2601

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INDEX WORDS

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SUMMARY

Five sterile hybrids (2n = 59) between a synthetic amphiploid of *Glycine tomentella* (2n = 38) and *G. canescens* (2n = 40) as female and soybean cultivars Lincoln and Hark as males have been produced by embryo or ovule culture using transplanted endosperm. The hybrid plants are twining perennials like the female parent but possess a number of morphological characters which reflect the presence of the soybean genome. Indophenol oxidase isozymes from leaf extracts also provide good evidence of the hybrid nature of the cultured plants. These hybrids open the way for the exploitation of the diverse germplasm resources of the perennial *Glycine* species in soybean breeding.

INTRODUCTION

The genus *Glycine* WILLD. to which the cultivated soybean belongs is currently divided into two subgenera, *Glycine* and *Soja* (MOENCH) F. J. HERM. The subgenus *soja* contains only cultivated soybeans *G. max* (L.) MERR. and its annual wild progenitor *G. soja* SIEB. and ZUCC. The subgenus *Glycine* contains seven perennial wild species all of which are indigenous to Australia (NEWELL & HYMOWITZ, 1980). Five occur only in Australia (*G. falcata* BENTH., *G. canescens* F. J. HERM., *G. latrobeana* (MEISSN.) BENTH., *G. clandestina* WILLD. and *G. latifolia* (BENTH.) (NEWELL & HYMOWITZ, 1978) while the remaining two species, *G. tabacina* (LABILL.) BENTH. and *G. tomentella* HAYATA, also occur throughout the Western Pacific region (BROUÉ, 1977; NEWELL & HYMOWITZ, 1980).

All the perennial species of *Glycine* are twining or scrambling herbs with a thick tap-root. They differ markedly in growth form and range of adaptation occupying habitats as diverse as the arid zone of central Australia, the alps of S.E. Australia and the tropical regions of northern Australia. As a result they possess many characteristics such as drought, heat and cold tolerance, apparent daylength insensitivity and disease resistance which if they could be transferred to cultivated soybeans would be of considerable economic importance (MARSHALL & BROUÉ, 1981; BURDON & MARSHALL, 1981a, b). The perennial *Glycine* species, therefore, represent a potentially valuable source of germplasm for soybean breeders.

However, to exploit this potential it is necessary to be able to hybridise the wild perennial *Glycine* species both among themselves and with the cultivated annual. In recent years substantial progress has been made in overcoming interspecific hybridisation barriers among the perennial *Glycine* species and we have succeeded in crossing each of the perennial species with at least one other (BROUÉ, et al., 1979). Attempts to hybridise the annual and perennial *Glycine* species have been far less successful. Indeed although fertilisation often occurs in crosses between species in subgenera *Glycine* and *Soja*, no fully developed seeds have been produced and no hybrid plants have previously been reported (NEWELL & HYMOWITZ, 1978; HYMOWITZ & NEWELL, 1979; LADIZINSKY et al., 1979).

Several fully developed hybrid plants between the perennial and annual *Glycine* species have recently been produced by *in vitro* culture of hybrid embryos obtained by pollinating complex amphiploids among the perennial species with soybean pollen. We describe the production, characteristics and implications of these hybrids here.

MATERIAL AND METHODS

Since the propensity to form interspecific hybrids is known to vary markedly both between species and between genotypes within a species in the genus *Glycine* (LADIZINSKI et al., 1979; BROUÉ et al., 1979) a wide range of species and genotypes were included in the initial crossing program. These included

- 11 lines of *G. max* or *G. soja;*
- 51 accessions representing the range of diversity in the seven perennial species;
- 24 intra- or inter-specific hybrids amongst the perennial *Glucine* species;
- 9 synthetic amphiploids derived by colchicine treatment of sterile interspecific hybrids among the perennial species.

The synthetic amphiploids were included in the program because of evidence in a wide range of species that interspecific crossability increases with increasing chromosome number in the female parent (HECTOR, 1936).

(i) Pollinations. Plants were grown and crossed in a temperature regulated greenhouse at Canberra, A.C.T. under natural daylength conditions. Initially, reciprocal crosses were attempted between soybeans and the perennial *Glycine* species. However, preliminary results indicated that the perennial species were easier to emasculate and pollinate and set a greater proportion of pods when used as female parent. Consequently in subsequent work, emphasis was given to the use of perennial species as females and soybeans as the male. The larger chasmogamous flowers borne in racemes were used in preference to the smaller, solitary cleistogamous flowers in making crosses. Emasculation and pollination were performed on the same day and all but one or two flowers were removed from each pollinated raceme to aid pod setting.

In the majority of cases, no pod growth was observed and the pollinated flowers dried up and fell off the plant in 3–4 days. However, in a small percentage of crosses ($\leq 13\%$ depending on the cross) pod initiation was observed. However, the embryos usually aborted between 5–35 days after pollination probably due to the degeneration of endosperm in the hybrids (LADIZINSKY et al., 1979) and no mature seed were obtained from the crosses.

(ii) Embryo and ovule culture. To facilitate the survival of interspecific hybrids, embryos or ovules were cultured on artificial media using a modification of the procedures developed by DE LATOUR et al., (1978) and WILLIAMS (1978). Pods were removed from the female parent 11–33 days after pollination and were surface sterilised for 10 minutes in a 1:1 mixture of 3% hypochlorite/1% aqueous cetrimide. They were then washed thoroughly in distilled water and dissected under sterile conditions. If the ovules in a pod were found to contain embryos large enough for dissection they were carefully removed from the embryo sac and placed with 'nurse endosperm' onto a small carrier square of filter paper, and thence to the surface of 3 ml of sterile nutrient medium in a 35 mm diameter plastic petri dish. If the embryo was considered too small and delicate to be removed from the ovule without damage the whole ovule was transferred to culture media without dissection. 'Nurse endosperm' was obtained from selfed seed of *G. max* (cv. Lincoln) 10–15 days after pollination. The composition of the nutrient media used throughout followed that of WILLIAMS (1978).

After transfer of embryos or ovules to the nutrient media the petri dishes were sealed with parafilm to prevent contamination and drying of the media. The petri dishes were kept at room temperature in the laboratory in diffuse light. Developing embryos were transferred to 19 mm diameter rimless test-tubes containing 8 ml sloped agar medium and 3 ml of liquid medium (half strength nutrients) when the unifoliate leaf was fully expanded and the primary root had formed visible root hairs. The tubes were stopped with non-absorbent cotton wool plugs and were kept in a 20 °C constant temperature room with 12 h day. When the seedlings had developed trifoliate leaves and possessed a rapidly developing branched root system they were transferred to small 50 mm diameter × 70 mm length tubes containing well washed vermiculite. These tubes were kept in a clear-lidded sealed plastic file box to prevent drying in a 20 °C constant temperature room and were watered on alternate days with half-strength Hoagland's solution and distilled water. Subsequently, the seedlings were transferred to sterile soil (3:1 sand/fertilised loam) and grown in a temperature regulated glasshouse.

Cytological and pollen fertility studies. Chromosome counts and associations were studied during meiosis in pollen mother cells. Young racemes were fixed in glacial acetic acid and absolute alcohol (1:3) for 25 hours and stored in 70% ethanol. Slides were prepared by squashing technique with 2% lacto-propanol orcein stain.

Pollen stainability of the putative hybrids was estimated by staining the anthers with aceto-carmine after fixation of flowers in acetic alcohol just prior to anthesis.

Verification of hybrids. The hybrid origin of the plants raised in tissue culture was verified by morphological comparison with parents for diagnostic traits such as flower colour, leaf shape and colour, electrophoretic comparison of leaf isozymes and by chromosome counts. Extracts for electrophoresis were prepared by grinding segments of fully expanded leaf lamina in three drops of 0.05 M phosphate buffer, pH 7.0, containing 2 mg/ml dithiothreitol. The crude extracts were absorbed on filter paper wicks (6 × 4 mm) which were then inserted in sample slots in a horizontal starch gel. Electrophoresis was carried out in two discontinuous buffer systems (Tris/citrate and Lithium/borate; see BROUÉ et al. 1977, for details). On completion of electrophoresis each gel was cut into three slices and the anodal portion of each slice was assayed

for a different enzyme. Leucine amino-peptidase (Lap), glutamate-oxalate transaminase (aspartate aminotransferase, Got) and NADH diaphorase (Nadhd) were detected on Lithium/borate gels, while esterase (Est), indophenol oxidase (IPO) and phosphoglucomutase (PGM) were detected on Tris/citrate gels. The staining procedures followed those given by BROUÉ et al. (1977) and BROWN et al. (1978).

RESULTS AND DISCUSSION

Over 2800 interspecific crosses were made between the perennial and annual *Glycine* species. Data on pod set following hybridisation, summarised in Table 1, confirm

Table 1. Interspecific crosses between perennial *Glycine* species and soybeans.

Female parent	Number of accessions	Number of crosses	Number of pods (%)
Species			
G. tomentella	16	824	45 (5.5)
G. canescens	9	303	7 (2.3)
G. tabacina	7	66	0 (0)
G. latrobeana	1	24	0 (0)
G. clandestina	2	13	0 (0)
G. falcata	2	36	0 (0)
G. latifolia	2	29	0 (0)
G. max	12	120	1 (0.8)
Intraspecific hybrids			
G. tomentella × *G. tomentella*	10	394	24 (6.1)
G. canescens × *G. canescens*	2	33	0 (0)
Interspecific hybrids			
G. tomentella × *G. canescens*	7	317	16 (5.1)
G. latrobeana × *G. canescens*	1	15	1 (6.7)
G. tabacina × *G. canescens*	2	20	0 (0)
G. falcata × *G. clandestina*	1	5	0 (0)
G. clandestina × *G. tomentella*	1	8	0 (0)
Amphiploids			
G. tomentella × *G. tomentella*	3	90	0 (0)
G. tomentella × *G. canescens*	4	703	55 (7.8)
G. tabacina × *G. canescens*	2	27	10 (3.7)

previous observations (LADIZINSKY et al., 1979; BROUÉ et al., 1979) that the different species of *Glycine* varied markedly in their propensity to form interspecific hybrids. *G. tomentella* set the greatest number of pods when pollinated with soybean pollen. LADIZINSKY et al. (1979) also found that male sterile Amsoy soybeans set the greater number of pods when pollinated with *G. tomentella* pollen. Taken together these results suggest that *G. tomentella* is the perennial progenitor of *G. soja* and hence, *G. max*.

The data in Table 1 also indicate that intra-specific and inter-specific hybrids of *G. tomentella* and *G. canescens* also form pods when pollinated by *G. max*. Synthetic

Table 2. Embryo and ovule culture of interspecific hybrids between perennial *Glycine* species and soybeans.

Female parent	Number of cultures[1]		Successful cultures[2]		Hybrid plants
	embryo	ovule	embryo	ovule	
Species					
G. tomentella	3	20	0	3	0
G. canescens	0	2	0	0	0
Intraspecific hybrids					
G. tomentella × *G. tomentella*	3	12	1	5	0
Interspecific hybrids					
G. latrobeana × *G. canescens*	0	1	0	1	0
Amphiploids					
G. tomentella × *G. canescens*	34	20	9	8	5
G. tabacina × *G. canescens*	1	0	0	0	0

[1] Number of pods from which embryos or ovules were cultured. Each pod usually contained more than one hybrid embryo.
[2] Culture is recorded as successful if it has produced a potted plant or one or more embryos are suriving in culture.

amphiploids between *G. tomentella* and *G. canescens*, the two most promiscuous species, produced more pods than either parent but the difference is small (7.8 versus 5.5 and 2.3 per cent, respectively).

The embryo and ovule culture data are summarised in Table 2. Embryos or ovules from a total of 96 pods were cultured. In most cases there was more than one hybrid embryo within a pod with an overall average of 2 embryos per pod. These yielded 27 successful cultures at the time of writing, five of which had produced potted plants. All five putative hybrids were from *G. tomentella* (accession number 1316, 2n = 38) × *G. canescens* (accession 1232, 2n = 40) amphiploid. In four cases the soybean cultivar Lincoln was the male parent, while cv. Hark was the male parent of the fifth putative hybrid.

All hybrids are highly sterile with 0 to 3 per cent stainable pollen. Limited pollinations with pollen from soybeans and selfed pollen have so far failed to produce seed. Preliminary cytological studies of chromosome associations during meiosis in pollen mother cells of one of the hybrids $(1316 \times 1232)^2 \times$ Lincoln indicated that the plant had 2n = 59 chromosomes as expected (Fig. 1) but meiosis was highly irregular with an average of 51.6 univalents and 3.9 bivalents/cell.

All putative hybrids were twining perennials and vegetatively resembled the female parent more than the male soybean (Fig. 2). However, they possessed a number of traits which strongly supported their hybrid origin including intermediate leaf shape (Fig. 3) and flower size and shape (Fig. 4). A comparative list of diagnostic morphological characters confirming the hybrid nature of these plants is given in Table 3.

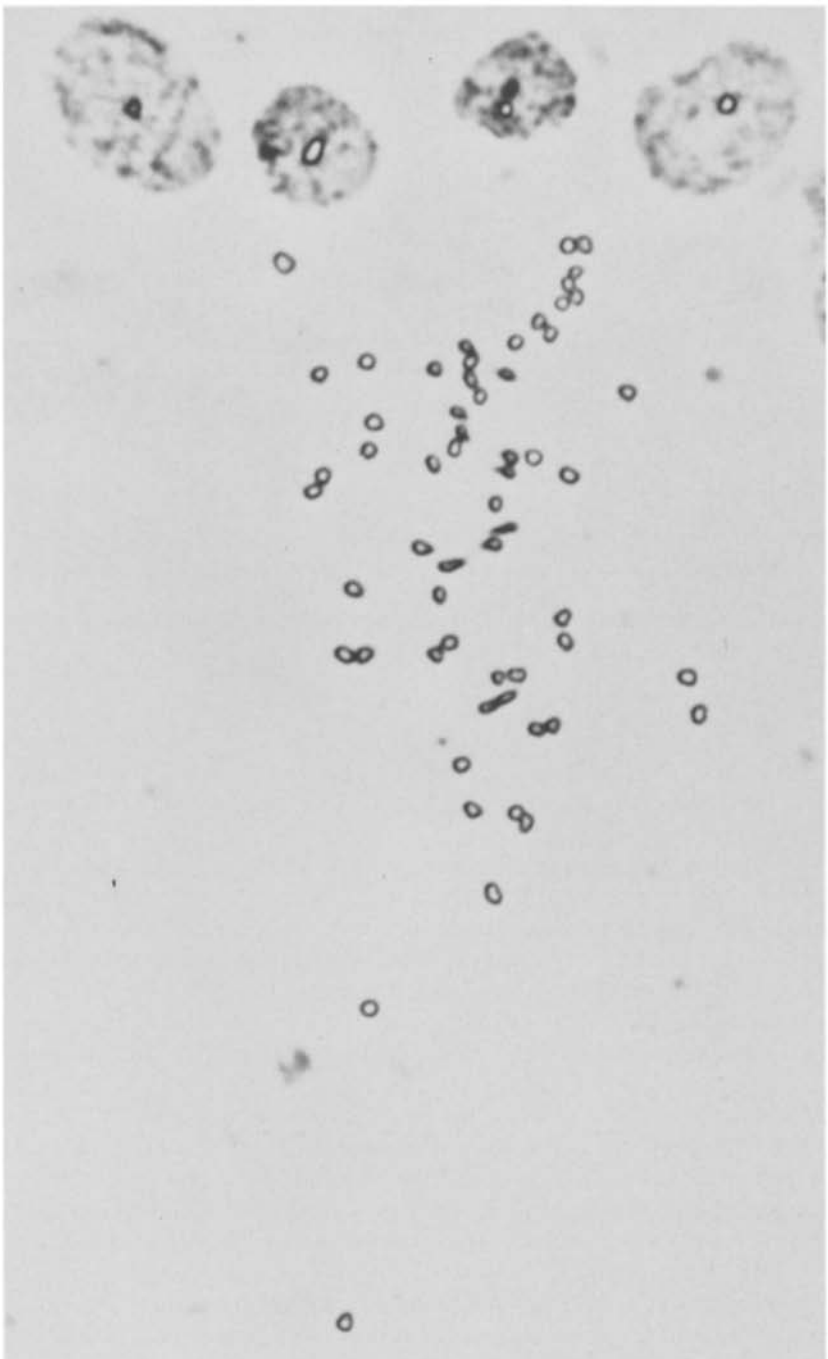

Fig. 1. Metaphase chromosomes of the hybrid [*G. tomentella* (1316) × *G. canescens* (1232)]^2 × *G. max* (Lincoln). 2n = 59; 53 I's and 3 II's.

Fig. 2. [*G. tomentella* × *G. canescens*]^2 × *G. max* hybrid (centre) compared with female amphiploid parent (right) and male soybean parent (left).

The leaf indophenol oxidase isozyme patterns also provided strong evidence of the hybrid origin of the plants derived by embryo culture. The putative hybrid possessed

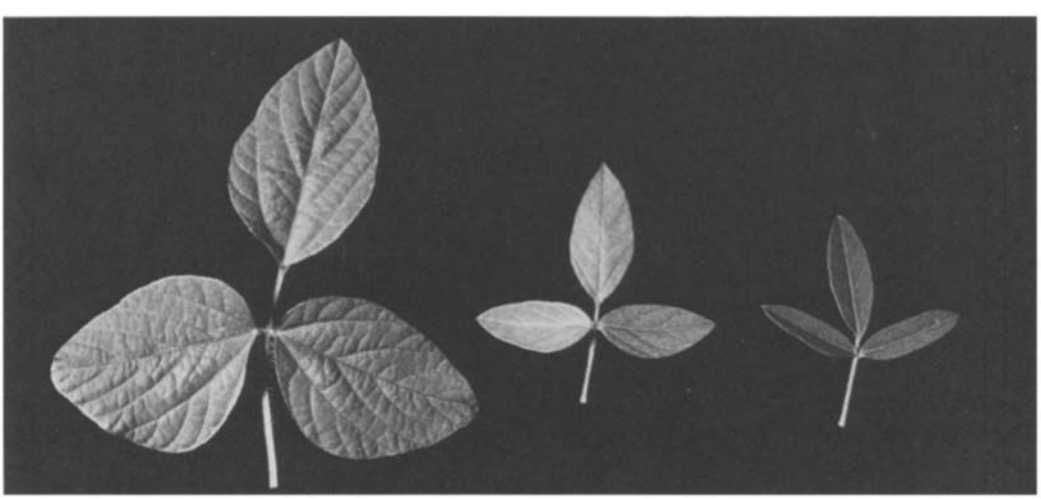

Fig. 3. Leaves of [*G. tomentella* × *G. canescens*]^2 × *G. max* hybrid (centre) compared to leaves of female amphiploid parent (right) and male soybean parent (left).

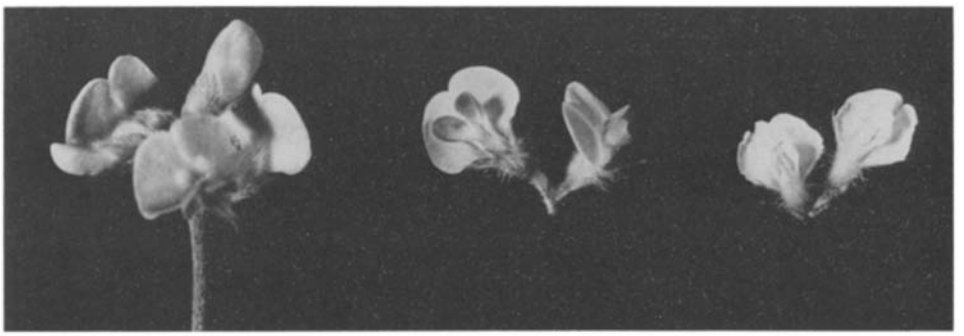

Fig. 4. Flowers of [*G. tomentella* × *G. canescens*]^2 × *G. max* hybrid (centre) compared with flowers of perennial female parent (left) and annual male parent cv. Lincoln (right).

Table 3. Comparative morphology of *G. tomentella* × *G. canescens* amphiploid, soybean cultivar Lincoln and their putative hybrid.

Attribute	Amphiploid	Hybrid	Lincoln
Flower colour	purple	pale lilac to pink	white
Flower size	large (11–12 mm)	intermediate (6–7 mm)	small (5–7 mm)
Inflorescence	raceme, 4–12 flowers	singly or pairs, subsessile	singly or pairs, subsessile
Floral bract	large (c 2 mm)	small (< 0.5 mm)	small (< 0.5 mm)
Terminal leaflet shape	elliptic	elliptic to ovate elliptic	broad elliptic to suborbicular
Stipules	narrowly triangular, densely pubescent	evenly triangular, pubescent	evenly triangular, pubescent
Upper leaf, pubescence	pubescent	intermediate	sparsely hirsute
Lower leaf, pubescence	hirsute, denser on all nerves	intermediate	openly hirsute on main nerves
Pubescence length	short (c 0.25 mm)	intermediate (c 0.7 mm)	long (1 mm)

a fast migrating band not present in the amphiploid but present in soybeans and a slower migrating band absent from soybeans but present in the amphiploid (Fig. 5). The other enzyme systems provided no information on the hybrid nature of these plants because all bands in soybeans were also present in the amphiploid.

The production of hybrids between annual and perennial *Glycine* species is an important first step towards exploiting the diverse germplasm resources represented by the wild perennial *Glycine* in soybean breeding. Further, they will enhance detailed cytogenetic studies of species affinities within and between the two subgenera of the genus *Glycine*. Studies are currently in progress to improve the efficiency of embryo culture techniques in this genus and to develop a wider range of hybrids between the annual and perennial *glycine* species.

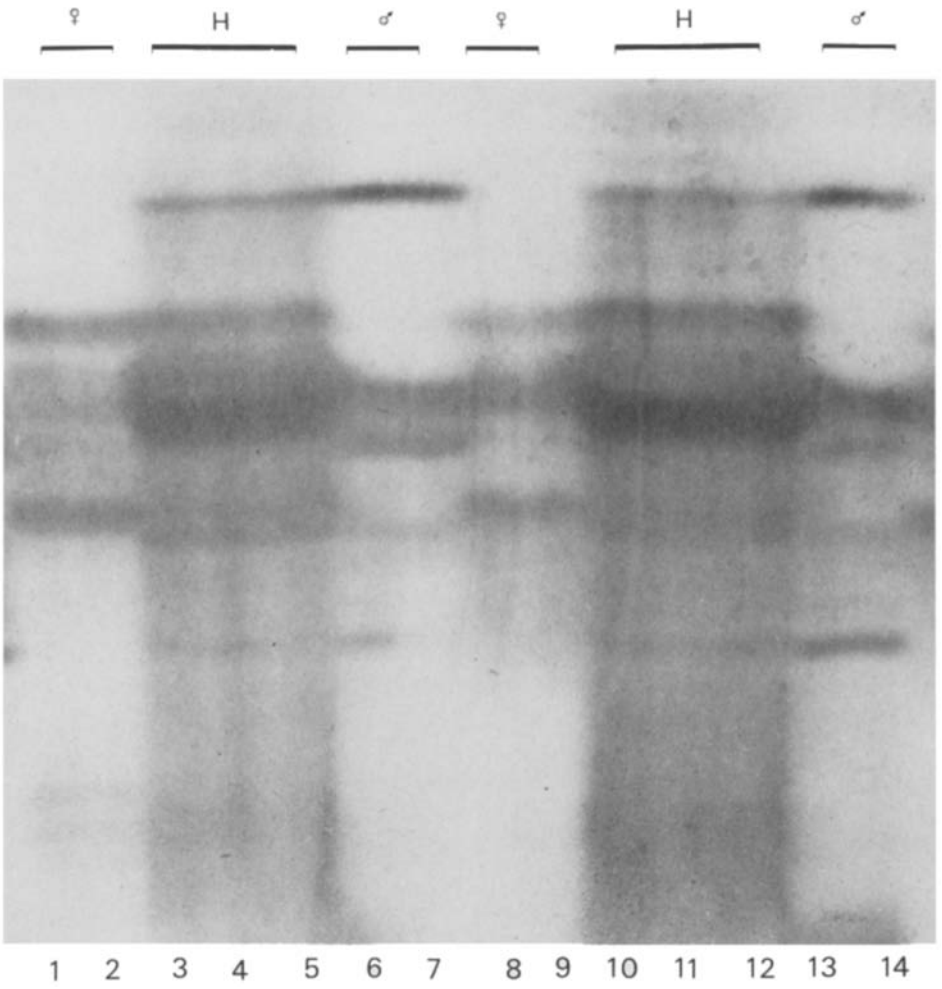

Fig. 5. Electrophoretic banding patterns of leaf indophenol oxidase isozymes of [*G. tomentella* × *G. canescens*]^2 × *G. max* (samples 3, 4, 5, 10, 11, 12), its perennial female parent (samples 1, 2, 8, 9) and annual male parent (cv. Lincoln, samples 6, 7, 13, 14).

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