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SHORT COMMUNICATION

Range of *Hevea brasiliensis* Pollen Dispersal Estimated by Esterase Isozyme Markers

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A study was carried out to estimate the distance *Hevea brasiliensis* pollen could be dispersed under natural conditions. Seeds were collected at varying distances from the boundary between two adjacent fields that were each planted with a pure stand of a genetic clone. Esterase isozyme markers were used to determine if the seeds had been derived from self- or cross-pollination. The incidence of cross-pollination was then examined in relation to the distance from the inter-clonal boundary. A logarithmic model gave the best fit ($r^2 = 0{\cdot}864$) and suggested that pollen could travel distances in the order of 0·3 to 1·1 km.

Key words: *Hevea brasiliensis* (rubber tree), pollen dispersal, cross-pollination, self-pollination, esterase isozymes.

INTRODUCTION

The commercial rubber tree, *Hevea brasiliensis* (Willd. ex A. Juss.) Mueller-Argoviensis, is an outbreeding monoecious species (Simmonds, 1986; Sunderasan *et al.*, 1994) that is pollinated by midges and thrips (Warmke, 1951, 1952; Rao, 1961). There is no information available on how far *Hevea* pollen can generally be dispersed by these pollinating agents in the course of open pollination. Such information on pollen travel can have useful applications in both *Hevea* research and cultivation. For example, this knowledge is important in the design and location of *Hevea* seed gardens. Elite seeds collected from isolated seed gardens give rise to trees of high vigour and latex yield that may be used in direct commercial planting as an alternative to vegetative propagation by bud-grafting. Knowing the range of pollen dispersal would enable a proper allocation of boundary allowances that would exclude or minimize pollination by extraneous pollen.

Concern for biosafety relating to gene escape during the field release of genetically transformed *Hevea* is another reason to obtain information on pollen travel. *Hevea brasiliensis* has been transformed (Arokiaraj *et al.*, 1994, 1998) and it is desirable that the initial field establishment of transgenic rubber plants be in areas distant from other *Hevea* plantings. Information on *Hevea* gene flow would facilitate the containment of transgenic *Hevea* to prevent the uncontrolled transfer of foreign or modified genes through natural pollination.

There are several approaches to using genetic markers to study gene flow through pollen dispersal. Most of these employ parental analysis to identify (usually male) parents and then quantify the pattern of gene movement using various algorithms (see the reviews on methods and data analysis software by Sork *et al.*, 1998). This paper describes a study using a single esterase isozyme marker to estimate the distance *Hevea brasiliensis* pollen is dispersed. Analysis of pollen dispersal was simplified by the selection of an experimental site consisting of two large adjacent fields, each planted with a single clonal cultivar of *Hevea*.

MATERIALS AND METHODS

Experimental area

Two large adjacent blocks of monoclonal rubber trees comprising 54 ha of the clone PR 107 and 70 ha of the clone RRIM 623 selected for the study satisfied the following criteria: (1) the adjacent fields were each planted with a genetically pure clonal stand; (2) isozyme markers were available for the clones in the two fields that enabled simple and unambiguous scoring of self- and cross-pollination; (3) the fields were sufficiently large in order that seeds could be collected from a number of stations located at varying distances from the inter-clonal boundary; (4) there was no other nearby source of extraneous *Hevea* pollen that might confound data interpretation. Trees planted 2×10 m apart in these fields were found to be clonally pure by trained inspectors on the basis of the clonal morphological characters of the trees. The experimental area was

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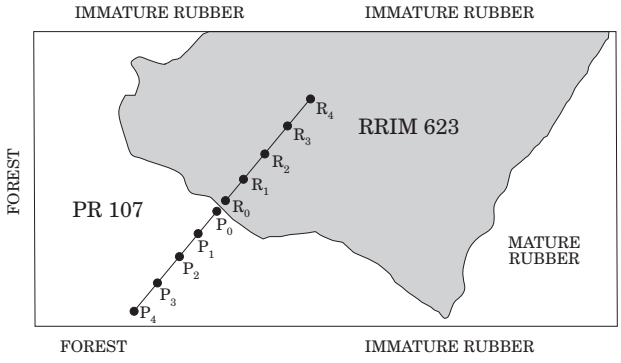

FIG. 1. Map of the experimental site and location of the seed collection stations. The experimental area is planted with the *Hevea brasiliensis* clones RRIM 623 and PR 107. The seed collection stations, R_0–R_4 in the RRIM 623 field and P_0–P_4 in the PR 107 field, are indicated. The distance between successive collection stations in a field is 100 m.

surrounded by immature (non-flowering) *Hevea* or by forest (Fig. 1). Mature rubber was planted across one other boundary of the RRIM 623 field, but this was at least 600 m away from where seeds were collected for this study.

Seed collection and germination

Ten seed collection stations were set up, five each in the RRIM 623 field and the PR 107 field. At each station, seeds were collected from a 4 m square fenced area. In each field, the stations were located 100 m apart along a straight line running at right angles to an approximately straight section of the inter-clonal boundary (Fig. 1). At the inter-clonal boundary, a stream running in between the clonal plantings gave rise to a buffer zone of 50 m separating the clones. Seeds were collected from the collection stations twice a week. The collected seeds were germinated and the seedlings maintained in a nursery.

Starch gel electrophoresis and esterase staining

Leaf tissue (0·5 g, midrib discarded) was ground in 1·5 ml of 0·07 M Tris-HCl buffer, pH 7·4, containing 10% w/v polyvinylpyrrolidone (PVP-40) in an ice-cold mortar. The homogenate was poured into microcentrifuge tubes and centrifuged for 30 min at 10000 *g* in a pre-chilled Sigma 201 M microcentrifuge. The clear supernatant was recovered and used for electrophoresis. Starch gel electrophoresis and isozyme staining were carried out according to Le Brun and Chevallier (1988). Leaf extracts (approx. 11 µl) were transferred on to 3·5 × 10 mm wicks of Whatman 1M filter paper and loaded on to 12% starch gels prepared in 0·1 M Tris-histidine buffer pH 6. Electrophoretic separation of the leaf proteins was carried out with 0·5 M Tris-citrate buffer in the refrigerator for about 5 h, at a constant current of 40 mA. Following electrophoresis, the gel was stained for esterase using α-naphthyl acetate in 0·1 M phosphate, pH 6 as the substrate and fast blue RR for isozyme detection. All reagents were from Sigma Chemical Co., USA.

RESULTS

As the two clonal plantings of RRIM 623 and PR 107 were 50 m apart, the furthermost stations were 450 m from the boundary. Seeds from RRIM 623 trees were collected in all five collection stations, but PR 107 seeds could only be recovered from the two stations nearest the inter-clonal boundary. The reason for the absence of seeds in the other stations could not be ascertained. As the area bordered the forest, interference by animals, possibly monkeys, was suspected. The number of seeds collected from each collection station is given in Table 1. Since the seeds were collected fresh, almost all seeds germinated successfully, giving an overall germination rate of 98·7%.

Esterase isozymes were used to determine whether the collected seeds had been derived from self- or cross-pollination. (Pollination in a tree that was effected by pollen from its genetic clone was also regarded as self-pollination, even though the pollen had originated from a separate tree. Essentially, these flowers were genetically selfed.) Of the major esterase bands that were observed in *Hevea* leaves, RRIM 623 was found to be homozygous for a slow-migrating esterase allele, whereas PR 107 was homozygous for a homologous fast-migrating allele (Fig. 2). Hence, both RRIM 623 and PR 107 progenies arising from self-pollination were readily identified by a single slow-migrating and fast-migrating band, respectively. On the other hand, progenies derived from cross-pollination were characterized by two bands: a slow-migrating allele having originated from the RRIM 623 parent and a fast-migrating allele from the PR 107 parent.

The incidences of self- and cross-pollination at the various seed collection stations in the RRIM 623 and PR 107 fields are depicted in Fig. 3. A good proportion (55·5%) of the RRIM 623 seeds was derived from self-pollination even at the inter-clonal boundary. Further away from the inter-clonal boundary and towards the centre of the field, the incidence of cross-pollination decreased as expected. Nevertheless, even as far as 450 m away from the boundary, 13·9% cross-pollination was still recorded in the RRIM 623 field. To estimate the maximum distance of pollen dispersal, the incidence of cross-pollination in RRIM 623 was examined in relation to the distance from the PR 107 field using either the original data or dispersal distance data that were logarithmically or exponentially transformed. A logarithmic model gave the best fit for the RRIM 623 seedlings ($r^2 = 0{\cdot}864$), suggesting that pollen could theoretically travel up to a distance of 1·1 km in this particular instance (Fig. 3).

The effectiveness of curve-fitting with data from the PR 107 seedlings was limited by the fact that seeds were only obtained from the two stations nearest the inter-clonal boundary, Stations 0 and 1 (Table 1). The proportions of cross-pollinations at the inter-clonal boundary (Station 0) were almost identical for RRIM 623 (46%) and PR 107 (48%) whereas cross-pollinations at Station 1 (32% and 21%, respectively) were somewhat more discrepant. To test for differences in the proportions of selfs and crosses between the clones for the two stations, isozyme data were fitted into 2 × 2 contingency tables for chi-square tests. The resulting χ^2 values showed no significant differences for

TABLE 1. *Seed collection from RRIM 623 and PR 107 fields*

RRIM 623 FIELD			PR 107 FIELD		
Seed collection station	Distance from boundary with PR 107 field (m)	Number of seeds collected	Seed collection station	Distance from boundary with RRIM 623 field (m)	Number of seeds collected
R_0	50	90	P_0	50	62
R_1	150	25	P_1	150	100
R_2	250	54	P_2	250	1
R_3	350	69	P_3	350	0
R_4	450	65	P_4	450	0

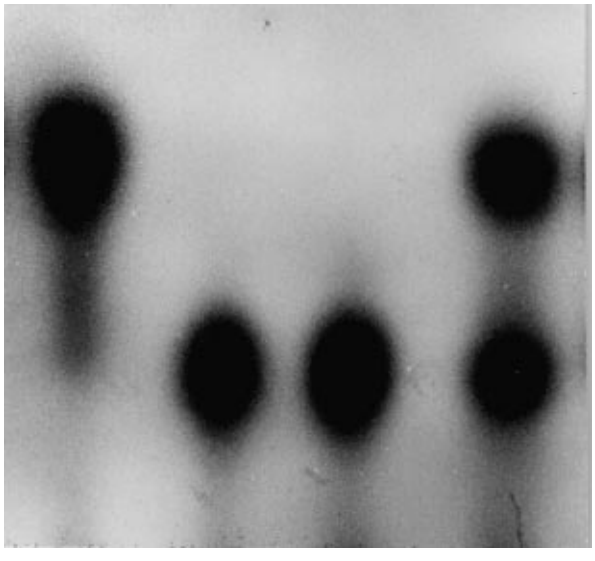

FIG. 2. Esterase isozymes from leaves of the parental clones RRIM 623 and PR 107 and from their progenies germinated from RRIM 623 seeds.

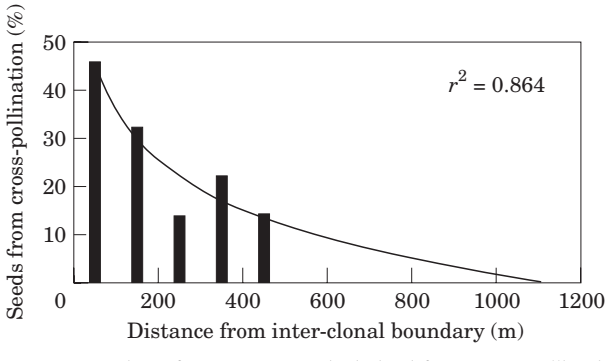

FIG. 3. Proportion of RRIM 623 seeds derived from cross-pollination in relation to the distance of their collection stations from the PR 107 field. Bars represent observed values. The curve is the logarithmic fit of the observed values.

both Station 0 and Station 1. This indicated that the proportions of crosses and selfs at the two PR 107 stations were consistent with those found for corresponding stations in the RRIM 623 field. The similarity served as a rationale to predict what the trend in pollen dispersal in the PR 107 field might be if it were governed by the same equation as that for the RRIM 623 field. Fitting the logarithmic model (as was done for RRIM 623 field), the dispersal distance RRIM 623 pollen in the PR 107 field was calculated to be 0·35 km. Hence, when the results of both the clones were taken into account, a preliminary estimate of *Hevea* pollen dispersal distance was in the order of 0·3 to 1·1 km.

DISCUSSION

The results on pollen dispersal distances presented are based on one experimental site, and further confirmatory studies should be undertaken in the future. Nevertheless, the experimental site provided an unusual opportunity to study pollen dispersal by simple isozyme tracking, without the involvement of multilocus allelic arrays or the need for complicated mathematical algorithms. Whereas many studies using genetic markers rely to some degree on paternity exclusion probabilities, paternity is unequivocal in the present case.

The efficiency of pollen dispersal is important in sexual propagation of *Hevea* as results from the available studies indicate *Hevea* is an outcrossing species. Based on the occurrence of yellow recessive mutants of the clone PB 5/51, Simmonds (1986) estimated the outcrossing rate of the clone planted in an estate to be between 72 and 84 %. More recently, the outcrossing rate of *Hevea* was estimated at about 60 % from isozyme analysis of the progenies of 'rogue' trees (i.e. trees of a discrepant clone) growing in the midst of otherwise pure clonal stands of estate rubber (Sunderasan *et al.*, 1994). In the present study, the female flowers—even those at the inter-clonal boundary—could be cross-pollinated only with pollen originating from the adjacent field, with the nearest trees 50 m away. Even so, 45 and 48 % cross-pollination was observed, respectively, for RRIM 623 and PR 107 trees growing at the inter-clonal boundary, affirming the tendency of *Hevea* towards outcrossing.

Pollination between different parental combinations could result in different proportions of selfs/crosses because of differing sexual compatibility, flowering synchrony, etc. In the case of the clones RRIM 623 and PR 107, Wycherley (1971) found their propensity to fruit-set to be very similar when selfed (2·0 and 1·8 %, respectively). Nevertheless,

fruit-set success *per se* is not expected to affect the estimate of pollen dispersal distance greatly. Results from this study showed that the proportion of crosses and selfs changed with the distance from the inter-clonal boundary and that this change could be described by a logarithmic curve. Hence, pollen dispersal was estimated by the *change in proportion* of the crosses and selfs with respect to the distance from the inter-clonal boundary. There is no reason to expect the pollen dispersal distance (controlled mainly by pollinating agents) to vary greatly when the pollination success changes or when the fruit-set success changes.

The results from this study showed that *Hevea* pollen could be transported fairly considerable distances by pollinating agents. The regression model in this study suggested a theoretical range of in the order 0·3 to 1·1 km, although very few pollen grains would be expected to reach this limit. This figure may be used as a basic guide to determine buffer boundaries that would exclude either the transfer of extraneous *Hevea* pollen into an isolated seed garden or the transfer of pollen from transgenic plants outside of the controlled planting.

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