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Realized pollen flow and wildling establishment from a genetically modified eucalypt field trial in Southeastern Brazil

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ABSTRACT

Confined field trials of genetically modified (GM) trees are the essential step toward the identification of the most productive cultivars and the assessment of the likely environmental impacts of the GM trees including the potential for gene flow by pollen and distance dispersal by seeds. Our study investigated the potential for wildling establishment and realized pollen flow from a clonal GM eucalypt field trial in Southeastern Brazil. The GM eucalypt stand was established in 2009, surrounded by a 3 m wide forest road and signal grass (*Bracharia* sp.) fields. No seedling regeneration was found between 2010 and 2014 in and around the stand, confirming the expectations of the unlikelihood of eucalypt seedling establishment based on its limited invasive potential in competitive tropical environments. In 2014, open-pollinated seeds were collected from 28 non-GM eucalypts located between three and 650 m distance from the GM trial. A total of 420 seedlings were grown in a greenhouse and screened for the presence of the transgenic construct by a multiplexed PCR assay targeting two transgenes and an internal control. The highest average transgene pollen flow (16%) was seen at short distances (3–15 m), rapidly diminishing to 3% by a 240 m distance and continued at this low rate up to the furthest distance assessed (650 m) from the GM trial. The negative exponential distribution of GM pollination success was similar to that observed in non-GM eucalypt pollen flow studies, indicating that low levels of effective pollination are expected over long distances. To the best of our knowledge, this is the first experimental assessments of realized pollen flow measured by the effective production of seedlings from a genetically modified tree in field conditions.

1. Introduction

Genetic modification (GM) has been proposed as a complementary technology to conventional and genomic assisted breeding to increase biomass production of intensively managed planted forests with potential for producing more wood on less land (Dubouzet et al., 2013; Harfouche et al., 2011). Forest productivity may also be increased by improving biotic and abiotic stress tolerance. Examples of GM trees expressing abiotic stress-tolerance (Harfouche et al., 2011; Osakabe et al., 2012) and enhanced disease resistance (Haggman et al., 2013) have been reviewed. Of special interest have been the cases of genetically engineered resistance to the Dutch elm disease in American elm and chestnut blight resistance in American chestnut (Thompson, 2012). Nevertheless, the deployment of genetically engineered trees has been a controversial issue (Brunner et al., 2007; Haggman et al., 2013). Some concerns put forward about potential damages to the environment have resulted in regulatory, certification and market obstacles, significantly hindering not only the commercial adoption of the technology but also the development of associated science that comes along (Strauss et al., 2015).

Confined field trials are small-scale field experiments carried out to evaluate the performance of genetically modified (GM) trees, providing the key experimental platform to advance their evaluation beyond the laboratory or greenhouse tests. Such trials enable scientists to evaluate the performance of GM material and collect

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data to meet regulatory requirements, including the potential for gene flow by pollen and distance dispersal by seeds, one of the main concerns raised for long lived forest trees (Brunner et al., 2007; Haggman et al., 2013). Such trials also allow the evaluation of the effectiveness of buffer strips around GM tree trials in reducing pollen flow and seedling establishment by seed dispersal outside the trial. Gene flow from tree plantations is one of the paramount processes affecting surrounding cross-compatible plantations and natural populations (DiFazio et al., 2012). This issue becomes significant with the development of GM trees for variable objectives such as disease and pest resistance, wood quality, drought tolerance and fast growth. The potential effects of gene flow by pollen and seed from GM trees to natural or other non-GM planted stands will depend, however, on the traits introduced and the species under scrutiny. Many genes of interest for commercial purposes are likely to present no or very low risk, either because they are very similar to native genes, or because they will be neutral or even reduce tree fitness (Brunner et al., 2007). Furthermore, this issue becomes more relevant when GM trees are deployed in regions that coincide with the center of origin and diversity of the species. When used as exotics, the expected impacts of GM trees on the native flora should be less of a concern due to the absence of naturally occurring cross-compatible forest populations.

Eucalypt trees are the most widely planted hardwoods in the world due to their outstanding ability to adapt, grow and provide quality wood for multiple applications (Myburg et al., 2007; Grattapaglia et al., 2012; Gonçalves et al., 2013). Due to their key role in modern intensive forestry worldwide, they have been an important target for genetic engineering efforts aimed at improving growth, wood quality and frost tolerance. A number of studies have assessed the potential seed-mediated gene flow from planted eucalypts into native forests in Australia in the context of 'genetic pollution' concerns (Barbour et al., 2010; Larcombe et al., 2014). The risk of eucalypt invasiveness by seed-mediated dispersal in exotic environments has also been investigated (Booth, 2012; Callaham et al., 2013; Calviño-Cancela and Rubido-Bará, 2013; Catry et al., 2015; Lorentz and Minogue, 2015; Silva et al., 2016). Pollen-mediated gene flow studies using molecular markers have been carried out mostly in the context of assessing the quality of seed orchard crops and the contamination potential of external pollen sources (Chaix et al., 2003; Grosser et al., 2010) without any particular concern with regard to the distance of gene flow. Early studies of pollen dispersal in *E. saligna* plantations in Brazil were based on tracking radioactive labeled pollen, revealing effective bee movement up to 100 m from their hives, with gradually decreasing activity up to a 300 m distance (Pacheco et al., 1986). Recently, we have investigated pollen-mediated flow and realized reproductive success at increasing distances from the source, across fragmented clonal stands of eucalypts in Brazil by parentage analysis of seedlings using microsatellite markers. We showed that pollination will take place by and large at relatively short distances of less than 200 m, consistent with the expected range of flight of pollinator bees (Silva et al., 2015). Going beyond the measurement of gene flow in natural stands, a spatially explicit landscape model to simulate pollination, dispersal, establishment and mortality, was proposed for Poplar in the US, showing that modeled transgene flow is highly context dependent and influenced by the competitive effect of transgenes, fertility of transgenic trees, plantation rotation length and patterns of selection (DiFazio et al., 2012).

While results of pollen mediated, gene flow studies in eucalypts clearly indicate that pollination will take place up to reasonably limited distances, pollen containment strategies by introducing sterility genes have been considered. Complete prevention of pollen production in greater than 95% of independently transformed lines was demonstrated in large-scale and multiple-year field tests of eucalypts by genetic modification with a pine male cone-specific promoter, PrMC2, driving a modified barnase coding sequences (Zhang et al., 2012). No studies to date, however, have reported experimental assessments of effective pollen dispersal from GM trees in field conditions. Given the recent Brazilian approval for commercial release of the first GM eucalypt in the world (Nature, 2015), such information should be relevant to complement existing data from molecular marker based pollen dispersal reports. In this study, we investigated wildling seedlings establishment and pollen dispersal from a contained field trial of GM eucalypt in Brazil.

2. Material and methods

2.1. Experimental design and sample collection

Our study was carried out in a location in Paranapanema, São Paulo state, in southeastern Brazil (23°35′S, 48°03′W, 670 m of altitude). The area included an experimental GM eucalypt stand established in 2009 using standard silvicultural practices as described earlier (Gonçalves et al., 2013). The experiment involved 1836 trees out of which 1665 were GM and 171 non-GM controls, in a 2.7 × 2.7 m spacing. Following the regulatory requirements by CTNBio (Brazilian National Technical Commission for Biosafety) (Normative Resolution No. 6, November 6th, 2008), the trial was surrounded by six border rows of a single non-GM clone and followed by a *Brachiaria* (signalgrass) field extending up to about 100 m from the forest stand, except where a non-GM eucalypt plantation had been previously established. To investigate realized transgenic pollen dispersal, open-pollinated seeds were collected from non-GM eucalypt trees including trees in the border rows as well as trees inside an adjacent non-GM eucalypt stand. Seeds were sampled from trees at seven distances from the GM tree stand: 3, 15, 220, 240, 260, 435 and 650 m (Fig. 1). At each distance, seeds from four randomly selected trees were collected totaling 28 trees. Open pollinated seeds from each sampled tree were planted in a greenhouse and 15 seedlings were randomly sampled per tree for genetic analysis. In total 420 seedlings were analyzed for the presence of transgene sequences. Leaf samples were collected for DNA analysis when the seedlings were 30 days old. Both GM trees and non-GM seed trees are hybrid clones of *Eucalyptus urophylla* × *Eucalyptus grandis*. Outside the experimental GM trial wildling establishment of GM seedlings up to 100 m from the stand was monitored every three months from 2010 to 2014, starting when the eucalypts initiated seed production (approximately 18 months after planting).

2.2. Detection of transgenic sequences

Total genomic DNA extraction was performed using a standard CTAB-based mini-prep protocol as described earlier for *Eucalyptus* (Grattapaglia and Sederoff, 1994). Two independent DNA extractions were carried out for each seedling on separate days to provide fully replicated results for each seedling to prevent the possibility of labeling error. DNA extractions were carried out in a laminar flow hood to prevent DNA cross contamination among samples. Transgenic sequence detection consisted in the PCR amplification of two specific genomic regions of the inserted transgenic construct. Primers were designed aiming at the detection of PCR products with sizes between 100 and 200 bp. Primer pairs were designed targeting two separate transgenic sequences, the neomycin phosphotransferase II gene (nptII) (forward primer: 5′-CAATAGCAGCCAGTCCCTTC-3′ and reverse primer: 5′ATGACTGGG CACAACAGACA-3′), and the nopaline synthase terminator (nos-terminator) from *Agrobacterium tumefaciens* (forward primer 5′-G