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Evaluation of efficiency and contamination rates of different controlled pollination methods in *Eucalyptus*

Avaliação da eficiência e taxa de contaminação de diferentes métodos de polinização controlada em *Eucalyptus*

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Resumo

O eucalipto é uma essência florestal muito utilizada para a produção de diversos tipos de produtos. A polinização controlada é um método empregado em programas de melhoramento que pode conferir resultados na primeira geração para caracteres desejados, como por exemplo, crescimento e produtividade. Na procura por redução de custos e melhora na eficiência, pesquisas sobre diferentes técnicas de polinização controlada são necessárias. O objetivo deste trabalho foi comparar quatro diferentes métodos de polinização controlada (convencional; *one-stop pollination* - OSP; OSP com saturação de pólen - OSPPS; e OSP com utilização de inseticida - OSPI) em relação à contaminação por pólen exógeno, ao percentual de retenção da cápsula e rendimento operacional. A taxa de retenção da cápsula e o rendimento operacional foram estatisticamente analisados e as análises de contaminações foram realizadas por meio de marcadores microssatélites. Todos os métodos mostraram-se eficiêncientes para evitar a contaminação por pólen exógeno. A polinização controlada convencional e a OSPPS tiveram os melhores percentuais de retenção da cápsula. Os métodos OSPPS e OSPI demonstraram os melhores resultados para no rendimento operacional. De acordo com nossos resultados, o método OSPPS seria o mais recomendado, pois apresentou melhores resultados em todas as avaliações.

Palavras-chave: Polinização controlada, contaminação do pólen, marcadores microssatélites, rendimento operacional.

Abstract

Eucalypts are widely used to produce various kinds of products. Controlled pollination methods used in breeding programs can produce desirable characteristics, such as growth and productivity, in the first generation. Considering the continuing need for cost reduction and improved efficiency, research on the various controlled pollination techniques is needed. The objective of this study was to compare four different techniques of controlled pollination (conventional; one-stop pollination - OSP; OSP with pollen saturation - OSPPS; and OSP with insecticide - OSPI) in terms of pollen contamination, percentage of capsule retention, and operational performance. Capsule retention and operational performance were analyzed statistically and contamination analyses were performed using microsatellite markers. All tested pollination methods proved efficient in avoiding contamination. The conventional controlled pollination and the OSPPS had the highest percentage of capsule retention. The OSPPS and OSPI methods obtained better results for operational performance compared to conventional controlled pollination and OSP. Based on our results, the OSPPS method is recommended because it produced the best results for all evaluated parameters.

Keywords: Controlled pollination, pollen contamination, microsatelites markers, operational performance.

INTRODUCTION

The *Eucalyptus* genus, belonging to the Myrtaceae family, has more than 900 species and varieties (Brooker; Kleinig, 2004; Boland et al., 2006). The vast majority of the genus originates from Australia, but there are two endemic species from New Guinea (*E. deglupta*) and Timor (*E. urophylla*) (LADIGES et al., 2003; BROOKER; KLEINIG, 2004). According to Eldridge (1975), the genus presents phenotypic plasticity and as such is cultivated widely around the world. It is the most extensively used plant in forestry worldwide, with 5.10 million ha alone planted with the genus in Brazil (ABRAF, 2013; GONÇALVES et al., 2013).

Eucalyptus has a mixed mating system with preferential outcrossing (GRIFFIN et al., 1987; PATTERSON et al., 2004). Therefore, forestry

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genetic improvement programs have begun to use controlled pollination as an important tool to maximize gains and provide superior genotypes more quickly than with open pollination (PATTERSON et al., 2004; SILVA et al., 2012). Conventional controlled pollination in *Eucalyptus* requires three visits; as such, several studies have developed alternative controlled pollination methods in order to increase efficiency and reduce labor costs (HARBARD et al., 1999; WILLIAMS et al., 1999; PATTERSON et al., 2004; ASSIS et al., 2005). Two such alternative pollination techniques are single-visit pollination (SVP; Williams et al., 1999) and one-stop pollination (OSP; HARBARD et al., 1999). These techniques have revolutionized controlled crossing in eucalypts because the techniques require only one visit to achieve pollination (Harbard et al., 1999; Williams et al., 1999; Patterson et al., 2004).

In choosing the controlled pollination technique, breeders consider the number of seeds produced, seed viability, operational performance and other aspects (Shelbourne, 1963; Libby et al., 1972; Hodgson, 1976; HARBARD et al., 1999; WILLIAMS et al., 1999). Another important factor is the level of pollen contamination (SHELBOURNE, 1963; PATTERSON et al., 2004; ASSIS et al., 2005; SILVA et al., 2012). In this context, the aim of this study was to evaluate the efficiency and contamination levels for several controlled pollination methods.

MATERIAL AND METHODS

Plant material

Four *Eucalyptus* clones, about 4.5 years old, from the clonal orchard of the Fibria Celulose SA company were used in the study. Two clones were *Eucalyptus grandis* species (G01 and G02) and two clones were hybrids of *Eucalyptus grandis* and *Eucalyptus urophylla* (H03 and H04). Controlled crosses were performed between the two *Eucalyptus grandis* clones (G01 x G02) and between the two hybrids (H03 x H04) using four pollination methods, totaling eight treatments. The parents G01 and G02 and 25 100-day-old progeny plants from each pollination method were selected for microsatellite marker analysis.

Pollination methods

Controlled pollinations were performed in the field over two periods: during the middle of the flowering period (March) and at the end of the flowering period (June). Pollen in quantities of 0.1 g per 100 flowers was used in conventional controlled pollination, one-stop pollination (OSP), and OSP with insecticide application (OSPI). For the OSP with pollen saturation method (OSPPS), 0.3 g of pollen per 100 flowers was used. Pollination was performed in a total of 10,063 closed mature flowers (with operculum). We then assessed the percentage of capsule retention and the number of seeds produced per fruit using Tukey's test in the Statistical Analysis System (SAS) software (SAS INSTITUTE, 2002), with a statistical significance determined at $p<0.05$. To avoid contamination, we removed the open flowers near the pollinated flowers. One hundred seeds from each treatment were planted and grown in a greenhouse, completely randomized in 5 blocks with 20 plants.

Conventional controlled pollination

After rinsing the flowers, we removed the operculum, and the flowers were emasculated according to Van Wyk (1972). The emasculated flowers were then bagged to avoid pollinator activity (HODGSON, 1976; VAN WYK, 1972). Three to five days after the removal of the operculum, we pollinated the flowers using a paintbrush and the flowers were again isolated. On the third visit, about two weeks after the pollination, the flowers were debagged (SHELBOURNE, 1963).

One-stop pollination (OSP)

The OSP method consists of applying the pollen immediately after emasculation (HARBARD et al., 1999) which followed the same procedure as the conventional controlled pollination method. After emasulation, we transected the stigma tip and the pollen was applied. The pollinated flowers were isolated using a plastic tube supported on the stigma (HARBARD et al., 1999).

Two OSP derivative methods, developed by the forestry tree improvement team of Fibria Celulose SA company, were also performed: 1) OSPPS: after emasculation, we manually applied triple the amount of pollen than the other methods and did not use the plastic tube isolation; 2) OSPI: Autan® insect repellent was applied using a nebulizer before the removal of the opened flowers and after emasculation.

DNA extraction

Genomic DNA was isolated from leaves using the CTAB method proposed by Murray and Thompson (1980) with modifications. About 150 mg of plant material were grounded in liquid nitrogen to a fine powder. The powder was homogenized in 700 µL of CTAB extraction buffer (2% CTAB; 2% PVP; 100 mM Tris-HCl pH=8.0; 25 mM EDTA; 2 M NaCl; 0.2% 2-mercaptoethanol) and heated for 45 min at 65°C. 600 µL of chloroform-isoamyl alcohol (CIA 24:1) was then added and the mixture was centrifuged for 5 min at 13,400 g. The upper phase was transferred to a new tube, 100 µL of 5% CTAB solution (5% CTAB; 2% PVP; 100 mM Tris-HCl pH=8.0; 25 mM EDTA; 2 M NaCl) was added, and a new extraction with CIA was carried out. The upper phase was transferred to a new tube and 400 µL of chilled isopropyl alcohol was added and the mixture incubated overnight at -20°C. The samples were then centrifuged for 5 min at 13,400 g, the supernatant discarded, and the pellet washed with 95% ethanol. The DNA was dissolved in 80 µL TE buffer (pH=8.0) containing 10 ng/µL of RNAse.

Microsatellite marker analysis

The primer pairs EMBRA05, EMBRA06, and EMBRA08, developed by Brondani et al. (1998), were used in the microsatellite analysis. The primers EMBRA05 and EMBRA08 were labeled with fluorochrome 6-FAM, and the primer EMBRA06 was labeled with fluorochrome NED. PCR consisted of 10 ng DNA, 1 µL 10X PCR Buffer (Invitrogen), $MgCl_2$ 1 mM, 1.25 µL BSA 10 mg/mL (Bovine Serine Albumin - Invitrogen), 1 mM dNTP mix, 0.45 µM of primers, 1U of Taq DNA polymerase (Invitrogen), and ultrapure water up to final volume of 11.5 µL. The reaction was performed under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min. Subsequently, 2 µL of the PCR product was diluted in 18 µL of water and 1 µL of the diluted PCR product was added to 10 µL solution of formamide and fragment size standard labeled with ROX (49:1). After solution denaturation in 95°C for 5 min, PCR products were placed on an ABI 3100 DNA analyzer (Applied Biosystems) for automated capillary electrophoresis. Genotyping data of parents and progenies (25 plants from each pollination method) were analyzed using the GeneScan/ Genotyper software (Applied Biosystems).

RESULTS AND DISCUSSION

Capsule retention

Interspecific hybridizations can occur naturally within the *Eucalyptus* genus. *Eucalypt* breeding programs use this hybridization capacity as a strategy to generate cloning candidates with high productivity and adaptability (ZOBEL ; Talbert, 1984; FERREIRA, 1992; ROCKWOOD , 2012; Gonçalves et al., 2013). However, flowering homogeneity presents a difficulty for improvement programs since different eucalypt species exhibit distinct flowering periods (SILVA et al., 2012). Therefore, we performed the controlled pollination during two distinct periods based on the flowering of the chosen species.

The crosses between *E. grandis* species were performed in the middle of the flowering period of the species, whereas the interspecific crosses (*E. grandis* x *E. urophylla*) were performed at the end of the flowering period of *E. grandis*. The pollinations performed during the middle and end of the flowering period showed statistically similar results (Table 1) with a slight increase in pollination at the end of the flowering period, indicating that the flowering period does not have an impact on fruit yield.

Due to environmental differences between the cardinal directions, such as incidence of light or wind, we evaluated whether direction has an effect on the percentage of capsule retention. Similar to the results for flowering period, there was no difference in harvested fruit relative to the cardinal direction (Table 2). In other words, environmental differences between cardinal orientations are not significant enough to affect fruit production.

Table 1. Percentage of capsule retention during the two flowering periods.
Tabela 1. Porcentagem de retenção da cápsula nos dois períodos florais.

Flowering period	Cross	Number of pollinated flowers	Number of retained capsules	Capsule retention (%)
Middle of flowering period (March)	G01 x G02	6,216	2,401	38.6 a
End of flowering period (June)	H03 x H04	3,847	1,698	44.1 a

Significant differences are indicated with lowercase letters ($p < 0.05$)

Table 2. Percentage of capsule retention relative to cardinal direction of the branch.
Tabela 2. Porcentagem de retenção da cápsula em relação à direção cardeal do ramo.

Cardinal direction	Number of pollinated flowers	Number of retained capsules	Capsule retention (%)
East	1,853	685	37 a
North	2,764	1,274	46.1 a
West	3,219	1,390	43.2 a
South	2,227	750	40.7 a

Significant differences are indicated with lowercase letters ($p < 0.05$)

The OSP method is an alternative pollination technique considered more advantageous than the conventional method because it involves only one pollination visit (Harbard et al., 1999; WILLIAMS et al., 1999; PATTERSON et al., 2004). This technique is used in plantations worldwide, including Brazil, for breeding and to mass-produce seeds of elite crosses (PATTERSON et al., 2004; ASSIS et al., 2005; Silva et al., 2012). Beyond the conventional and OSP methods, two techniques (OSPPS and OSPI) developed by Fibria Celulose SA company were also tested.

Among the assessed pollination techniques, the conventional and OSPPS methods produced the most favorable results (Table 3 and 4). In *E. grandis* crosses, the OSPPS method showed better efficiency than the conventional method, but in the hybrid crosses the best method was the conventional (Table 4). The OSPI method presented the poorest results (Table 3 and 4), with a reduction in efficiency of 47.9% in *E. grandis* crosses and 66.7% in hybrid crosses compared to the results for the bestmethod. The low capsule retention in OSPI (14.4%) suggests that some ingredient in Autan® insecticide, which contains alcohol, propane/butane, and diethyltoluamide (DEET; the active ingredient), may inhibit pollen germination or have a toxic effect on the flowers.

Assis et al. (2005) compared the conventional, OSP, and artificially induced protogyny (AIP) pollination methods. In their Brazilian trials, the results for the mean capsule retention using the conventional method (39.8%) was similar to our results (58.2%). However, the OSP results from Assis et al. (2005) showed a higher level of capsule retention (79.2%) compared to the 25.2% obtained in our study. The high level of capsule retention for OSP in the Assis et al. (2005) study may be explained by differences in methodology; they did not provide the pollen quantity used in pollination and the mother trees were exposed to a controlled dose of the growth retardant paclobutrazol.

Controlled pollination is laborious and expensive, requiring time and specialized professionals. As such, forestry companies are looking for pollination techniques with high operational efficiency and seed production. We measured operational efficiency by the number of flowers pollinated per person per hour and the number of staff per hour required to

Table 3. Percentage of capsule retention for each pollination method.
Tabela 3. Porcentagem da retenção da cápsula em cada método de polinização.

Pollination method	Number of pollinated flowers	Number of retained capsules	Capsule retention (%)
Conventional	2,809	1,635	58.2 a
OSP	2,476	625	25.2 b
OSP with pollen saturation	2,720	1,543	56.7 a
OSP with insecticide	2,058	296	14.4 c

Significant differences are indicated with lowercase letters ($p < 0.05$)

Tabela 4. Percentage of capsule retention for each treatment.
Table 4. Porcentagem de retenção da cápsula para cada tratamento.

Pollination method	Cross	Number of pollinated flowers	Number of retained capsules	Capsule retention (%)
Conventional	G01 x G02	1,554	690	44.4 b
	H03 x H04	1,255	945	75.3 a
OSP	G01 x G02	1,551	460	29.6 c
	H03 x H04	925	165	17.8 d
OSP with pollen saturation	G01 x G02	1,555	998	64.2 a
	H03 x H04	1,165	545	46.8 b
OSP with insecticide	G01 x G02	1,556	253	16.3 d
	H03 x H04	502	43	8.6 e

Significant differences are indicated with lowercase letters ($p < 0.05$)

pollinate 1,000 flowers. In the assessment of the number of staff per hour required to pollinate 1,000 flowers, we assume that the technique was performed by a team in which each person is responsible for one step of the pollination (emasculation, pollination, isolation, and identification). Common activities to all methods, such as displacement and fruit harvest, were not considered; we only took into account the time spent on the techniques separately.

The results demonstrated superior operational performance for techniques that do not require flower isolation (OSPPS and OSPI) compared to the techniques that use barriers to avoid pollen contamination (conventional and OSP) (Table 5). OSPPS and OSPI methods increased the operational performance by up to 2.8 times and 5.2 times in flowers/person/hour and number of staff/hour/1,000 flowers, respectively. Our results were similar to those found by Assis et al. (2005) which showed efficiency of 35 and 105 pollinated flowers per person per hour in conventional and OSP techniques, respectively.

Pollen contamination

Controlled pollination in the field is subject to pollen contamination of nearby trees due to the mixed mating system of the *Eucalyptus* genus (GRIFFIN et al., 1987; Patterson et al., 2004). Pollen contamination in *E. grandis* controlled crosses was analyzed using molecular marker genotyping with the microsatellite markers EMBRA05, EMBRA06, and EMBRA08 (supplementary material), with the pattern of expected progeny segregation shown in Table 6. Analysis of the allelic profiles from the parent trees and progeny (25 seedlings) showed consistency with the expected pattern for microsatellite markers EMBRA06 and EMBRA08 (supplementary material). In these markers, all 25 offspring possess alleles derived from both parents. However, the conventional and OSPI methods showed genotyping inconsistent with the expected pattern for EMBRA05 in one out of 25 analyzed offspring seedlings. In these seedlings, we observed the presence of a different fragment (alleles 103 and 121) (supplementary material), indicating contamination with exogenous pollen for these two pollination techniques.

Determining the level of contamination by non-target pollen is a key issue in controlled pollination methods. The low level of contamination in *E. grandis* crosses that we observed (5%) was similar to experiments with *Eucalyptus globulus* reported by Harbard et al. (2000), with a contamination rate of 4% using the OSP method. However, studies of contaminants in controlled crosses of *E. grandis* by OSP and AIP showed a higher contamination rate than our study, of 12% (HARBARD et al., 2000) and 7.6% (ASSIS et al., 2005), respectively. According to Harbard et al. (2000), a contamination rate of about 10% is considered acceptable for seed production.

CONCLUSIONS

Based on our results, the four tested pollination techniques are effective in avoiding pollen contamination for improvement programs and suitable for the mass-production of seeds of elite crosses. However, based on capsule retention and operational performance results, the OSP with pollen saturation was

Tabela 5. Number of flowers pollinated per person per hou and estimate of the number of staff per hour required to pollinate 1,000 flowers for each pollination method.
Table 5. Número de flores polinizadas por homem-hora e estimativa de número de homens-hora requeridos para processar 1.000 flores, para cada método de polinização controlada.

Pollination method	Flowers/person/hour	Number of staff/hour/1,000 flowers
Conventional	54 c	20.91 a
OSP	94 b	8.88 b
OSP with pollen saturation	150 a	3.99 c
OSP with insecticide	144 a	4.3 c

Significant differences are indicated with lowercase letters ($p < 0.05$)

Tabela 6. Segregation pattern expected in the *E. grandis* progeny based on the genotypes of parents (mother - G01; father - G02).
Table 6. Padrão de segregação esperado nas progênies baseado na genotipagem dos parentais (*E. grandis*, mãe - G01 e pai - G02).

Microsatellite marker	G01 alleles	G02 alleles	Expected progeny
EMBRA05	111/129	117/131	111/117; 111/131; 117/129; 129/131
EMBRA06	116/134	120/128	116/120; 116/128; 120/134; 128/134
EMBRA08	157/157	144/167	144/157; 157/167

considered the best method as the cost-benefit relationship was more favorable in comparison with the other methods.

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REFERENCES

ABRAF - BRAZILIAN ASSOCIATION OF FOREST PLANTATION PRODUCERS. **Statistical Yearbook 2013:** base year 2012. Brasília, 2013. 148 p.

ASSIS, T. F.; WARBURTON, P.; HARWOOD, C. Artificially induced protogyny: an advance in the controlled pollination of *Eucalyptus*. **Australian Forestry**, London, v. 68, n. 1, p. 27-33, 2005.

BOLAND, D. J.; BROKER, M. I. H.; CHIPPENDALE, G. M.; HALL, N.; HYLAND, B. P. M.; JOHNSON, R. D.; KLEINING, D. A.; MCDONALD, M. W.; TURNER, J. D. **Forest trees of Australia**, 5.ed. Collingwood: CSIRO Publishing, 2006. 768 p.

BRONDANI, R. P. V.; BRONDANI, C.; TARCHINI, R.; GRATTAPAGLIA, D. DEVELOPMENT, CHARACTERIZATION AND MAPPING OF MICROSATELLITE MARKERS IN *EUCALYPTUS GRANDIS* AND *E. UROPHYLLA*. **THEORETICAL AND APPLIED GENETICS**, HEIDELBERG, V. 97, P. 816-827, 1998.

BROOKER, M. I. H.; KLEINIG, D. A. **Field guide to *Eucalyptus***, 2.ed. Melbourne: Bloomings Books, 2004. v. 3, 400 p.

ELDRIDGE, K. G. **An annotated bibliography of genetic variation in *Eucalyptus camaldulensis***. Oxford: Commonwealth Forestry Institute, 1975. 59 p.

FERREIRA, M. Melhoramento e a silvicultura intensiva clonal. **IPEF**, Piracicaba, v. 45, p. 22-30, 1992.

GONÇALVES, J. L. M.; ALVARES, C. A.; HIGA, A. R.; SILVA, L. D.; ALFENAS, A. C.; STAHL, J.; FERRAZ, S. F. B.; LIMA, W. P.; BRANCALION, P. H. S.; HUBNER, A.; BOUILLET, J. P. D.; LACLAU, J. P.; NOUVELLON, Y.; EPRON, D. Integrating genetic and silvicultural strategies to minimize abiotic and biotic constraints in Brazilian eucalypt plantations. **Forest Ecology and Management**, Amsterdam, v. 301, p. 6–27, 2013.

GRIFFIN, A. R.; MORAN, G. F.; FRIPP, Y. J. Preferential Outcrossing in *Eucalyptus regnans* F. Muell. **Australian Journal of Botany**, Collingwood, v. 35, n. 4, p. 465-475, 1987.

HARBARD, J. L.; GRIFFIN, A. R.; ESPEJO, J. E. Mass controlled pollination of *Eucalyptus globulus*: a practical reality. **Canadian Journal of Forestry Research**, Ottawa, v. 29, n. 10, p. 1457-1463, 1999.

HARBARD, J. L.; GRIFFIN, A. R.; ESPEJO, J. E.; CENTURION, C.; RUSSELL, J. 'One stop pollination' a new technology developed by Shell Forestry Technology Unit. In: DUNGEY, H. S.; DIETERS, M. J.; NIKLES, D. O. (Eds). Hybrid Breeding and Genetics of Forest Trees. 2000. Queensland. **Proceedings…** Queensland: QFRI/CRC-SPF, 2000. p. 430-434.

HODGSON, L. M. Some aspects of flowering and reproductive behavior in *Eucalyptus grandis* (Hill) Maiden at JDM Keet Forest Research Station. **South African Forestry Journal**, Pretoria, v. 97, p. 18-28, 1976.

LADIGES, P. Y.; UDOVICIC, F.; NELSON, G. Australian biogeographical connections and the phylogeny of large genera in the plant family Myrtaceae. **Journal of Biogeography**, Malden, v. 30, n. 7, p. 989-998, 2003.

LIBBY, W. J.; KIANG, Y. T.; LINHART, Y. B. Control pollination seeds from cuttings of coast redwood. **Silvae Genetica**, Frankfurt, v. 21, p. 17-20, 1972.

MURRAY, M. G.; THOMPSON, W. F. Rapid isolation of high molecular weight plant DNA. **Nucleic Acid Research**, Oxford, v. 8, n. 19, p. 4321-4325, 1980.

PATTERSON, B.; GORE, P.; POTTS, B. M.; VAILLANCOURT, R. E. Advances in pollination techniques for large-scale seed production in *Eucalyptus globulus*. **Australian Journal of Botany,** Collingwood, v. 52, n. 6, p. 781-788, 2004.

ROCKWOOD, D. L. History and Status of Eucalyptus Improvement in Florida. **International Journal of Forestry Research**, New York, 2012.

SAS Institute. **SAS user's guide**: statistics. Cary: SAS Institute, 2002.

SHELBOURNE, C. J. A. Controlled pollination work *with Eucalyptus grandis*: selfing, crossing and hybridizing with *Eucalyptus tereticornis*. **Forest Research Pamphlet**, Kitwe, n. 7, p. 4, 1963.

SILVA, P. H. M.; MORAES, C. B.; MORI, E. S. Polinização controlada em eucaliptos nas empresas florestais brasileiras. **Circular Técnica IPEF**, Piracicaba, v. 204, p. 1-12, 2012.

VAN WYK, G. Report on pollen handling control pollination and grafting of *Eucalyptus*. **South African Forestry Journal**, Pretoria, v. 101, p. 47-53, 1972.

WILLIAMS, D. R.; POTTS, B. M.; BLACK, P. G. Testing single visit pollination procedures for *Eucalyptus globulus* and *E. nitens*. **Australian Forestry**, Queen Victoria, v. 62, n. 4, p. 346-352, 1999.

ZOBEL, B.; TALBERT, J. **Applied forest tree improvement**. New York: John Wiley and Sons, 1984. 505 p.

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