Therefore, we estimated five separate indices (CSP_{i1} to CSP_{i5}) that combine stability and performance simultaneously. All of these indices were very effective to detect genotypes with both desirable stability and performance levels. Among the five indices CSP_{i5} was the best index both in its predictive power and in its applicability.

Acknowledgement

The authors gratefully acknowledge the contributions from the following persons and institutions: JOSEPH SVOLBA helped in the establishment and maintenance of the test sites; Drs. MANFRED HUEHN, TORE SKRØPPE, JÖRG KLEINSCHMIT and CSABA MATYAS provided valuable comments on the manuscript. ALEXANDER VON HUMBOLDT Foundation in Bonn provided research support to KANI ISIK during the evaluation of the study at the LSFRI, Dept of Forest Genetic Resources, Escherode, Germany.

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Inducing Male Flowering by Applying Gibberellic Acid has no Effect on the Cry j 1 Content in *Cryptomeria japonica* Pollen

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(Received 22nd January 2003)

Abstract

Cryptomeria japonica pollinosis has recently become a serious problem in Japan. Two major allergens of *C. japonica* pollinosis, Cry j 1 and Cry j 2, have been isolated and characterized. Cry j 1 and Cry j 2 are basic proteins with molecular weights of 41-46 kDa and 37 kDa, respectively, and it was reported that more than 90% of *C. japonica* pollinosis patients had IgE specific to both of them. Several studies have found large variations in the content of Cry j 1, a major allergen of *C. japonica* pollinosis, suggesting that pollinosis could be reduced by replacing current *C. japonica* varieties with trees that produce less Cry j 1. In this study, Cry j 1 contents were compared in pollen produced with and without inducing male flowering by applying gibberellic acid (GA), which is a very useful technique for stimulating pollen production in targeted trees. No effect of

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GA application was detected, showing that pollen collected from GA-treated trees could be used in further investigations of the pollen's variability in Cry j 1 contents. GA application could also help in screening programs to identify trees that produce pollen with low allergen contents from candidates that generate sparse amounts of pollen. Furthermore, intraclonal variability of Cry j 1 content was found to be very low compared to interclonal variability. The stability of Cry j 1 content within clones suggests that selecting trees that produce less Cry j 1, followed by mass production via vegetative propagation, offers great potential for reducing the serious health problems caused by pollinosis in Japan.

Key words: Cry j 1, *Cryptomeria japonica,* pollinosis, gibberellic acid, clonal repeatability.

Introduction

Cryptomeria japonica D. Don (Sugi) is one of the most important tree species in Japan, especially for timber production. It is favored for its straight bole and rapid growth, and it covers about 4.5 million ha, accounting for 45% of the artificial forest in Japan. Afforestation with *C. japonica* trees mainly occurred in the 1950's ~ 1960's, and these trees have now fully matured to develop flowers and release abundant pollen. The first report that *C. japonica* pollen causes a seasonal allergic disease was that of HORIGUTI and SAITO (1963). Since then, the number of *C. japonica* pollinosis patients has increased, climbing to more than 10 % of the Japanese population. This has been exacerbated by changes in the environment such as air pollution (TAKAFUJI and NAKAGAWA, 2000), and the life style of Japanese people, such as increases in the fat and protein intake (NAGAKURA, 1998). *C. japonica* pollinosis is thus a serious social problem and reduction of pollinosis has become a priority for the authorities. One proposed measure is to select *C. japonica* trees that produce less pollen, and this approach is being actively pursued by the Japanese Forestry Agency. Male sterile trees have also been found by TAIRA *et al.* (1993) and it was revealed that male sterility is a heritable character (TAIRA *et al.,* 1999). These male sterility trees also would be used for reducing pollinosis in future.

Two major allergens of *C. japonica* pollinosis, Cry j 1 and Cry j 2, have been isolated and characterized (YASUEDA *et al.,* 1983; TANIAI *et al.,* 1988; GRIFFITH *et al.,* 1993; SONE *et al.,* 1994; SAKAGUCHI *et al.,* 1990; NAMBA *et al.,* 1994). Cry j 1 is a basic glycoprotein with a molecular weight of 41–46 kDa and pI 8.9-9.2, showing pectate lyase activity, and Cry j 2 is a basic protein of 37 kDa and pI 9.5, which has polygalacturonase activity. HASHIMOTO et al. (1995) reported that more than 90% of *C. pollinosis* patients had specific IgE to both allergens, and the remainder had specific IgE to either one of them. Recently, the content of Cry j 1 and Cry j 2 in pollen has been found to vary among trees. We also discovered that the Cry j 1 content varied from 16–1320 µg per gram pollen among 158 plus trees selected in Kanto Breeding Region (GOTO *et al.,* 1999). This finding suggests that trees producing low amounts of the allergen could also be bred as a means of reducing pollinosis. However, regardless of whether such trees were to be used directly, or for breeding less allergenic trees, the inheritance and clonal repeatability of the allergen content in the pollen have to be investigated. In such studies, it would be necessary to collect pollen samples from targeted trees consistently over several years. However, the abundance of male flowers produced differs among trees subjected to varying levels of solar radiation, precipitation and warmth in July, when differentiation of the male flowers starts (YOKOYAMA and KANAZASHI, 1994). The most desirable trees for reducing pollinosis would be those producing low amounts of pollen with low levels of allergen. For the selection of such trees, it is important to collect pollen samples from trees producing pollen sparsely, although such trees tend not to set male flowers in "off" years. Thus, artificial flower induction is required for reliable determination of the allergen content in pollen. Gibberellic acid (GA) is an endogenous plant hormone known to be associated with flower differentiation of many tree species (PHARIS, 1985), including *C. japonica* (KATO *et al.,* 1958; HASHIZUME, 1959). In fact, GA application is essential for seed production and artificial crossing of *C. japonica.* GA application could also be helpful for studies of allergen contents, and selection of less allergenic trees, provided its application does not affect allergen content. In this study, we investigated the effect of GA application on Cry j 1 content in the pollen of *C. japonica.* In addition, we examined the intraclonal and interclonal variability of the Cry j 1 content in pollen because these are important criteria for the practical use of trees that produce low levels of the allergens in afforestation.

Materials and Methods

Pollen samples

Pollen samples were collected from eight plus trees selected in Kanto Breeding Region (Kuji 2, Kuji 3, Kuji 18, Kuji20, Kuji 37, Naka 3, Taga 4 and Tsukuba 2), growing at two seed orchards which are located next to each other (Nos. 1 and 6 of the Ibaraki Prefectural Government Forestry Technology Center, Naka, Ibaraki) in March 2000. The seed orchards Nos 1 and 6 were established in 1970 and 1992, respectively, and trees growing at No. 6 had never been treated with GA. Therefore, trees growing at No.6 were used as control. On the other hand, each trees growing at seed orchard No. 1 were treated with GA by placing two CMC (carboxymethylcellulose, Sunrose, Nippon Paper Chemicals Co. Ltd., Japan) pastes containing 2.5 mg of GA (Kyowa Hakko Kogyo Co., Ltd., Japan), under the bark of their trunks in July 1999 (AKUZAWA, 1988). Following this, the bark was taped to keep the CMC paste in place. At each seed orchard, pollen samples were collected from 2 ~ 6 ramets for each of the eight clones. The number of ramets for each clone and seed orchard used in this study is shown in *Table 1.* For pollen sample collection, branches setting male flowers were cut, covered with paper pollination bags, placed with their bases in water in February 2000, and the anthers were allowed to open. After pollen shedding, the pollen samples were collected from the bag and screened using gauze.

Cry j 1 extraction

Pollen samples were collected and desiccated in a tight box containing silica gel at 4°C for a month and then stored at –30°C prior to Cry j 1 extraction. Cry j 1 was extracted from 100 mg of pollen samples with 5 ml of 0.125 M $NaHCO_3$ (pH

Table 1. – Number of ramets for each clone used in this study. Treated clones were from Seed Orchard No. 1, and controls were from Seed Orchard No. 6.

clone	treated	control
Kuji 2	4	5
Kuji 3	5	3
Kuji 18	5	5
Kuji 20	2	4
Kuji 37	5	5
Naka 3	6	3
Taga 4	5	5
Tsukuba 2	5	4
total	37	34

8.3) at 4°C, for 2 h, following the method of SAWATANI *et al.* (1993).

Measurement of Cry j 1 using monoclonal antibody-based ELISA

Measurement of Cry j 1 was performed by sandwich ELISA using two monoclonal antibodies, J1B01 and J1B07 (SUZUKI *et al.,* 1996). For this, a polystyrene microtitre plate (MaxiSorp, Nalge Nunc Int., Rochester, NY USA) was coated with 200 ng of J1B07 in 100 µl of 0.1 M carbonate buffer (pH 9.6), overnight at 4°C. The plate was washed four times with PBS (phosphate-buffered saline) containing 0.1% Tween 20 (w/v, PBS-T) and incubated with PBS containing 1% bovine serum albumin (w/v, PBS-T-BSA) for 1h at 37°C. After washing four times with PBS-T, 100 µl of either Cry j 1 standards or 1:400–1:25600 dilutions of pollen extracts in PBS-T-BSA were added to the wells and incubated for 2 h at 37°C. The plate was then washed four times with PBS-T and incubated with 50 ng of biotinylated J1B01 in 100 µl of PBS-T-BSA for 2h at 37°C. After washing four times with PBS-T, the plate was incubated with 0.03 U of streptavidin-ß-galactosidase conjugate (Roche Diagnostics, Mannheim, Germany) in 100 µl of PBS-T-BSA at 37°C for 1h and then washed four times. Finally, 100 µl of 5mM o-nitrophenyl-ß-D-galactopyranoside in 0.02 M NaH_2PO_4 (pH 7.2) was added to the wells and incubated at 37°C for 1 h. The enzyme reaction was then stopped with 100 µl of 1 M Na_2CO_3, and the absorbance at 415 nm was measured using a microplate reader (Model 550, Bio-Rad Lab. Inc., Hercules, CA USA). Microplate manager II software (Bio-Rad) was used for calculating the concentration of Cry j 1.

Statistical analysis

The effect of GA application on Cry j 1 content was tested by ANOVA (analysis of variance) using StatView v. 4.5 (Abacus Concepts, Inc. Berkeley, CA USA). The clonal repeatability (R) of Cry j 1 content was estimated from the ANOVA results using the following formula.

$R = V_G/V_P = \sigma_c^2/(\sigma_e^2 + \sigma_{ct}^2 + \sigma_c^2)$

where; V_G = genotypic variance

V_p = phenotypic variance

σ_c^2 = variance among clones

σ_{ct}^2 = variance of clone x treatment interaction

σ_e^2 = error variance (variance among trees within clones)

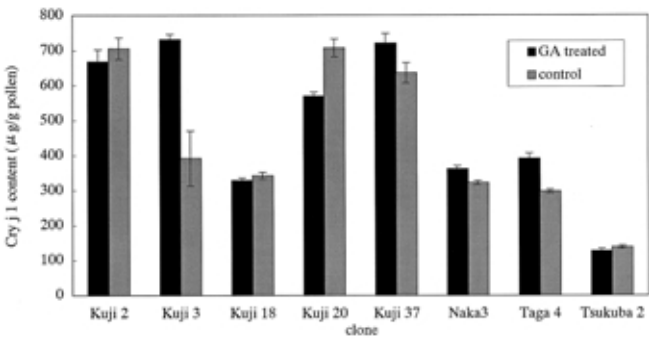

Figure 1. – The interclonal variation and the effect of GA treatment on Cry j 1 content. The vertical bars indicate standard error.

Results

Clonal variation and the effect of GA application on Cry j 1 content

The mean Cry j 1 content of each clone treated with GA and the untreated controls is shown in *Fig. 1.* The results of the ANOVA test are given in *Table 2.* Cry j 1 content varied from 130 to 687 µg per gram pollen among clones and significant differences were detected among clones in the ANOVA test ($p < 0.01$, *Table 2a*). On the other hand, no significant difference in Cry j 1 content was found between treated and untreated trees ($p > 0.05$, *Table 2a*), suggesting that GA application had no effect on the Cry j 1 content. However, a significant treatment x clone interaction was detected ($p < 0.01$, *Table 2a*), indicating that the response to GA application differed among clones. This was potentially important, because if the ranking of the clones is changed by GA application, the selection of trees that produce low amounts of Cry j 1 should be performed without application of GA. However, the mean Cry j 1 content in pollen samples of seven out of eight clones was similar between treated and untreated ramets. The only exception was clone Kuji 3, which showed a large difference between treated and untreated ramets. When data related to Kuji 3 were removed from the ANOVA test, no significant treatment x clone interaction was detected among the seven remaining clones ($p > 0.05$, *Table 2b*). This suggests that the treatment x clone interaction was caused by Kuji 3. Comparing the Cry j 1 contents of Kuji 3 ramets, it was found that the Cry j 1 content was similar among all the treated ramets and one of the untreated ramets, but the Cry j 1 contents of the other two untreated ramets were much lower than those of all the other Kuji 3 ramets (*Fig. 2*).

Clonal repeatability of Cry j 1 content in pollen

Clonal repeatability was evaluated from the ANOVA results from eight clones. The error variance, the variance of treatment x clone interaction and variance among clones were 0.009, 0.033 and 0.164, equivalent to 4.5%, 15.9% and 79.6% of phenotypic variance, respectively. The estimated clonal repeatability was 0.796.

Table 2 (a). – ANOVA results of the effects of treatment, clone and their interaction on Cry j 1 content in pollen using all eight clones. *(b)* ANOVA results using all clones except Kuji 3.

a

	df	MS	*F*	*p*	expected variance component
treatment	1	0.03166	3.413	0.701	
clone	7	0.36213	39.042	<0.0001	$\sigma_e^2+4.12\,\sigma_{ct}^2+8.24\,\sigma_c^2$
clone × treatment	7	0.03492	3.765	0.0021	$\sigma_e^2+4.12\,\sigma_{ct}^2$
error	55	0.00928			

b

	df	MS	*F*	*p*
treatment	1	0.00004	0.005	0.9427
clone	6	0.40679	58.161	<0.0001
clone × treatment	6	0.01085	1.551	0.1818
error	49	0.00699		

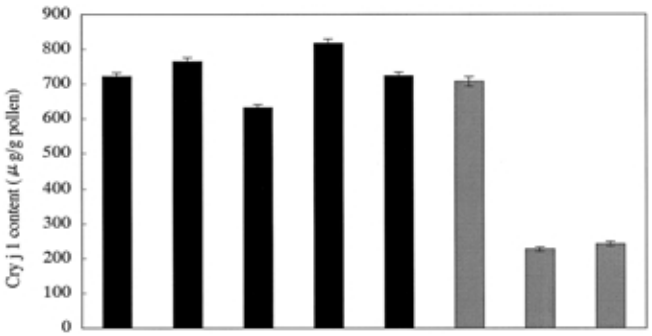

Figure 2. – The variation in Cry j 1 content in pollen among Kuji 3 ramets. The vertical bars indicate standard error.

Discussion

Large variations in Cry j 1 content among trees have been previously reported and the possibility of using trees that produce less Cry j 1 to reduce pollinosis has been suggested (SASAKI *et al.,* 1996; GOTO *et al.,* 1999; SAITO and TERANISHI, 2002). To select such trees, inducing male flowering by applying GA would be helpful since it allows pollen to be collected even from trees that produce pollen sparsely, or in "off" years. In this study, we compared Cry j 1 content between GA treated and untreated ramets to determine the effect of GA application on Cry j 1 content. Because it is unknown how long the effect of GA application remains, ramets which had never been treated with GA were used as control. GA treated ramets and controls were growing at different seed orchards namely Nos. 1 and 6, respectively. However, the site effect was not included because the two seed orchards are located closely each other in flat and open place.

SAITO *et al.* (2002) reported that GA application did not affect the Cry j 1 content per a pollen grain. The present study further supports the finding that GA application does not affect Cry j 1 content, thus allowing its use to induce male flowering in comparative studies of Cry j 1 content. A significant clone x treatment interaction was detected, but further analysis showed that the interaction was caused solely by one clone, Kuji 3. Amongst the remaining seven clones, the means and ranking of Cry j 1 contents were similar for treated and untreated ramets. Furthermore, comparison of the Cry j 1 content among individual trees of Kuji 3 indicated that the clone x treatment interaction was not caused by GA application. Thus, it should be possible to select trees that produce low amounts of Cry j 1 using GA-treated trees. RAPD analysis was performed on ramets of Kuji 3 to detect possible misplanting or mislabeling, as described by KANAYAMA *et al.* (2002). The results indicated that no misplanting or mislabeling had occurred with Kuji 3 (data not shown). Thus, the difference in micrometeorological factors or soil condition between the two seed orchards might affect the Cry j 1 content.

On the other hand, since seed orchard No. 1 was established in 1970, whereas No. 6 was set up in 1992, untreated ramets growing at No. 6 are 22 years younger than GA-treated ramets growing at No. 1. The difference in tree age might have caused the difference in Cry j 1 content. Further studies on the Cry j 1 content of the Kuji 3 ramets in subsequent years might clarify this issue. Generally, based on our results, we propose that selection of trees with less Cry j 1 should be carried out in two steps. Firstly, candidate trees that produce low amounts of Cry j should be screened from a large number of clones using one or a few trees per clone. Clones showing high intraclonal stability should then be retained from the candidates in a second step.

In an earlier paper, we reported that Cry j 1 content varied in the range 16 ~ 1320 µg per gram pollen among trees (GOTO *et al.,* 1999). In addition to the considerable variation among trees, Cry j 1 content was relatively constant among ramets of the same clone in this study. These two studies suggest there is sufficient interclonal variability and stability within clones to make exploitation of trees that produce low amounts of Cry j 1 a viable proposition. In an attempt to reduce pollinosis, trees that produce low levels of pollen (male flowers) have already been selected and used. Trees producing less pollen with less allergen per weight would help reduce pollinosis further. The abundance of male flowers differs considerably among clones (HASHIZUME, H., 1990; MASUDA *et al.,* 1993; TODA *et al.,* 1996; SENDA *et al.,* 1999) and the variation seems to be larger than that of Cry j 1 content, especially in "on" years. However, the clonal repeatability of Cry j 1 content is considerably higher than 0.076 and 0.106, those of male flower setting reported by MASUDA *et al.* (1993). In addition, compared to male flower setting, Cry j 1 content is easier to measure precisely. Therefore, the Cry j 1 content in pollen would be at least as valuable a target trait as male flower setting in attempts to produce trees with reduced allergenicity.

Another interesting finding of this study is that with the exception of Kuji 3, no significant difference was detected in Cry j 1 content between the two seed orchards, although trees growing at seed orchard No. 1 were 22 years older than those at No. 6. This result suggests that Cry j 1 content in pollen is similar even among clonal trees propagated in different years and thus supports the availability of less Cry j 1 trees in forestry. Moreover, Cry j 1 content may not change as the trees age, implying that juvenile selection would be possible for Cry j 1 content in pollen produced by inducing male flowering via GA applications. In conclusion, we propose that trees that produce less Cry j 1 should be selected, as a complement to trees that produce low amounts of pollen, to reduce pollinosis in Japan.

Acknowledgement

The authors are grateful to Ibaraki Prefectural Government Forestry Technology Center for help in collecting pollen samples and Dr. S. S. KAUNDAN for critical reading of the manuscript.

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Studies on Effect of Nutrient Media for Clonal Propagation of Superior Phenotypes of *Dalbergia sissoo* Roxb. through Tissue Culture

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(Received 3rd March 2003)

Abstract

Micropropagation studies were undertaken using nodal explants collected from two ramets (clone No. 36) belonging to 60-year-old superior tree of *Dalbergia sissoo.* Two nutrient media viz. MS and B5 were used to find out the suitability of the medium. Bud break was achieved in both of the media within 6-8 days under different media combinations supplemented with BAP (0.10–1.0 mg/l) alone as well as in combinations with IAA or NAA (0.10 to 0.50 mg/l). Maximum percentage of bud break (100%) was achieved in both of the media. Maximum number of shoots per explant (8.04) was observed in the MS medium supplemented with 1.0 mg/l BAP + 0.25 mg/l NAA. Out of the two nutrient media tried MS medium was found to be the best, which gave high rate of shoot proliferation as compared to B_5 medium. Maximum number of roots per plantlet (4.47) was observed in $^1/_2$ MS supplemented with (1.0 mg/l) IBA within 18 days. Plantlets so produced were acclimatized and transplanted to pots and 70% survival was achieved.

Key words: Dalbergia sissoo, Micropropagation, *In vitro*, Clonal Propagation.

Abbreviations: MS: MURASHIGE and SKOOG medium; B5: GAMBORG et al. medium; BAP: benzylamino purine; NAA: naphthalene acetic acid; IBA: indole-3-Butyric acid; IAA: indole-3-acetic acid; Kn: kinetin; PGR: Plant Growth Regulator.

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Introduction

Dalbergia sissoo is an indigenous, commercial timber yielding species of northern India. It is found up to 900 m in the sub-Himalayan tract and occasionally ascending to 1500 m between latitude 21.17° N to 32.60° N and longitude 74.8° E to 93.43° E. In the sub-Himalayan tract, it occurs along rivers and streams, gregariously growing on alluvial soil. It is a multipurpose nitrogen fixing tree, but its major end use is as wood, with grower and market appeal as a quality cabinet timber. Any breeding programme to improve wood quality must focus primarily on vigour and form. This is particularly for *D. sissoo*, which is characterized by crooked stem, forking and ramicorn branching. Trees showing straight bole are of rare occurrences. Early trials have shown that crooked stem form of *D. sissoo* is under strong genetic control and has high heritability 42–65% (VIDAKOVIC and AHSAN, 1970). There exists considerable phenotypic variation between trees of different provenances (SEWAL et al., 1988). If such superior phenotypes having desired qualitative and quantitative traits are multiplied vegetatively, tangible gains can be achieved. Conventional vegetative propagation methods using branch cuttings may result in plagiotrophic growth (Personal communication K. White cited by SEWAL et al., 1988). Furthermore, if cuttings are taken from mature trees the success achieved in rooting is not encouraging until and unless the tissue has been rejuvenated following hedging. In this context micropropagation of superior phenotypes may