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Clonal Variation of Eucalypts in Susceptibility to Bacterial Wilt Detected by Using Different Inoculation Methods

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(Received 23th May 2013)

Summary

Four inoculation methods were investigated for assessing the clonal variation of eucalypts in susceptibility to bacterial wilt (*Ralstonia solanacearum*). The results showed that these inoculation methods obviously differed in the disease infection process, clonal variation and clonal mean repeatability in susceptibility of stock materials inoculated. For each inoculation method, the clonal effect was consistently significant over the assessment period. Root-collar suspension injection method (RSI) yielded the highest relative clonal variation (0.67 ± 0.086) and clonal mean repeatability (0.92 ± 0.038) in both disease infected incidence and severity at the end of assessment, attributable to the enhanced genetic variation or low environment effect. For a given inoculation method, an early assessment time might exist for maximizing relative clonal variation or repeatability. It is desirable in breeding to adopt an inoculation method and/or efficient assessment time with high clonal variance component, which would in turn improve the efficiency of clonal screening.

Key words: Eucalypt, *Ralstonia solanacearum*, bacterial wilt, inoculation, clonal variation, repeatability.

Introduction

Bacterial wilt caused by *R. solanacearum* is a common bacterium disease on eucalypts and many other crop or wood species (BUDDENHAGEN, 1986; HAYWARD, 1991; ROUX *et al.,* 2001; OLD *et al.,* 2003; PEGG *et al.,* 2003; NAIDOO *et al.,* 2011). *R. solanacearum* is soil-borne, and affects eucalypt seedlings or cuttings and young trees by ways of injured roots, stem wounds or through stomata. Bacterial wilt could easily occur and develop under hot and wet conditions during typhoon season each year in the South China (WU and LIANG, 1988; ZHOU *et al.,* 2008). Affected trees are often scattered throughout

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DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

stands and show wilting, leaf drop, stem death and reduced growth rate (Old *et al.,* 2003). In few cases, it was also observed that bacterial wilt almost destroyed the whole young stands (Lin *et al.,* 1996; Qi, 2002). This disease is recognized as one of the most significant risks for commercial investment in eucalypt plantations in the South China (Zhou *et al.,* 2008).

Variation in susceptibility to *R. solanacearum* exists among eucalypt species as well as varieties or clones (Dianese and Dristig, 1993; Old *et al.,* 2003; Huang *et al.,* 2008). This is fundamental for screening and breeding eucalypt clones of resistance. Artificial inoculation is commonly used to test the susceptibility or resistance of host to a pathogen (Atibalentja and Eastburn, 1997; Fang, 1998; Shi *et al.,* 2000). Different pathogen inoculation methods are expected to yield distinct results of disease infection (Dainese and Dristig, 1993; Atibalentja and Eastburn, 1997; Shi *et al.,* 2000; Hüberli *et al.,* 2002; Ruz *et al.,* 2008). However, little effort was made to investigate the likely difference in key genetic parameters in disease infection traits among different inoculation methods, which would affect the effective screening of resistant clones as for other silvicultural traits like growth and wood properties (Borralho *et al.,* 1992; Zhang *et al.,* 2003; Isik *et al.,* 2005; Baltunis and Brawner, 2010). In this study, we employed four inoculation methods to test the susceptibility of eucalypt clones to bacterial wilt, and to investigate the clonal or genetic variation under different inoculation methods.

Materials and Methods

Stock species and clones

The stock materials used in the inoculation experiment included the following species or hybrids: *Eucalyptus urophylla* S.T. Blake, *E. grandis* W. Hill ex Maiden, *E. wetarensis* L.D. Pryor, *E. camaldulensis* Dehnh, *E. tereticornis* Smith, *E. uropphylla* S.T. Blake x *E. grandis* W. Hill ex Maiden, *E. grandis* W. Hill ex Maiden x E. *urophylla* S.T. Blake, *E. grandis* W. Hill ex Maiden x *E. tereticornis* Smith, *E. urophylla* S.T. Blake x *E. camaldulensis* Dehnh, and *E. saligna* Smith x *E. exerta* F.V. Muell. These species and/or hybrids have been identified for developing short-rotation (5–7 years) commercial plantations in the South China (Qi, 2002; Wei, 2012). Two kinds of clonal materials were used in this study, *i.e.* well-acclimated and uniform tube (3.1 x 13 cm) cuttings about 4 months old and 25 cm in height, and semi-lignified shoots of 1 year old pot-raised hedge plants. The rooted tube cuttings, qualified for field planting, went through a full nursery culture process of rooting and growing under conditions of controlled rooting hormone, sunlight, temperature, moisture and nutrition, and acclimation or hardening under conditions of natural sunlight, no-fertilizing and minimum water; the semi-lignified shoots of the hedge plants were "standardized" materials for production of rooted cuttings (Qi, 2002). As we did not have a balance structure among species and the pedigree information for the hybrids, we treated all species clones as cultivated varieties. In addition, all the clonal varieties included were early exclusively selected for growth purpose. Considering this fact and possibly no phenotypic and genetic correlation between eucalypt growth and bacterial wilt (Gan *et al.,* 2004), we cautiously assumed that these varieties were randomly sampled in terms of bacterial wilt susceptibility or resistance.

Pathogen and inoculum preparation

A single pathogen isolate was used, which in an early study was found most virulent among 11 isolates originating from the main planting regions of eucalypts in the South China (Luo *et al.,* 2013). The purified pathogen isolate with distilled water was maintained at 4 °C in refrigerator. Before inoculation, the pathogen was cultured and propagated on a BPY medium (beef extract 3.8 g, peptone 5.0 g, yeast extract 3.0 g, sugar 20 g, agar 15 g, K_2HPO_4 2.0 g, KH_2PO_4 0.5 g, $MgSO_4H_2O$ 0.25 g, distilled water 1,000 ml, pH = 7.0) at 30 °C for 36 hr, and diluted with distilled water as the inoculum (suspension) with a concentration of 3×10^8 cells/ml. This concentration or similarity was found appropriate for inoculation experiment of eucalypt bacterial wilt in some early studies (Dianese and Dristig, 1993; Gan *et al.,* 2004; Huang *et al.,* 2008; Luo *et al.,* 2013).

Inoculation

Four pathogen inoculation methods were considered in the experiment:

1) Cutting suspension irrigation (CSI): The rooted cuttings with soil were carefully pulled out from the tube containers (3.1 x 13 cm), replanted in larger plastic bag containers (6 x 15 cm) by filling sterile soil substrate (a mixture of 30% yellow earth and 70% dry peat moss in volume) around (trimmed the dead roots growing out of bottom of the containers before replanting if necessary), and fully irrigated with inoculum suspension in the morning once a day for the first three days in a row. Minor injury might occur to some hair roots during the replanting. The inoculated cuttings were kept moist for the first three days and regularly irrigated with clean water from the fourth day, as required. This method was similar to the conventional pathogen inoculation (Fang, 1998; Shi *et al.,* 2000).

2) Bare-root cutting suspension culture (BSC): The cuttings were carefully pulled out from the plastic tubes, the soil was carefully washed away, the roots were trimmed to 1.0 cm from the taproot axis and 5 cm from the root collar, and the root-trimmed cuttings were cultured in the 100 ml beakers (52 x 72 mm) filled with the inoculum suspension. From the fourth day, clean water was properly added if necessary.

3) Root-collar suspension injection (RSI): Before inoculation, the plastic tube cuttings were fully sprayed with clean water. The inoculum suspension was well injected with a sterile injector at the root-collar of each cutting once a day for the first three days in a row. The inoculated cuttings were regularly irrigated with clean water as required.

4) Shoot suspension culture (SSC): The uniform semi-lignified shoots with a length of about 30 cm were first collected from the pot-raised hedge plants, and were

DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

Table 1. – Criteria for rating the disease severity of bacterial wilt after inoculation.

Class	Score	Criteria of wilting (flabby and drooping)
1	0	Health and vigorous, no wilting symptom
2	1	Leaf tip wilting on 2~3 leaves
3	2	Leaf wilting on 1/4~1/2 leaves
4	3	Leaf wilting on 1/2~3/4 leaves
5	4	Leaf wilting on more than 3/4~all leaves or dead

then trimmed to a length of 22 cm. Leaves at lower part (about 8 cm length) were removed. The trimmed shoots were cultured in glass tubes filled with the inoculum suspension. From the fourth day, clean water was properly added if necessary. This method or equivalent was early adopted for quick testing and screening of resistant or susceptible eucalypt varieties (WANG *et al.,* 2011; LUO *et al.,* 2013).

One factor (clone) experiment design with multi-replicates was adopted for each inoculation method. In the experiment, four inoculation methods were separately applied to test 20 clones each with 6 replicates (cutting or shoots). The same set of 20 clones were tested with three inoculation methods (CSI, BSC and RSI), of which 8 clones along with other 12 clones were tested with SSC. Therefore, there were totally 32 clones included in the experiment.

The inoculation work was carried out in the morning of the 9th and the next two days of August 2006. All inoculated cuttings by using CSI and RSI were placed into greenhouse with day-time temperature more than 30 °C and relative humidity more than 70%. The inoculated materials by using BSC and SSC, respectively, were kept in lab with day-time temperature more than 28 °C (LUO *et al.,* 2013). The inoculated materials were maintained under the conditions as described above over a period by the end of which a stable disease symptom was reached.

Disease symptom assessment

Disease severity (D_S) was rated for each cutting or shoot on a scale of 0 to 4 (*Table 1*). Disease development was assessed on a daily basis for BSC and SSC, but daily for the first 10 days and at 2-day interval from the 10th day for CSI and RSI after inoculation. The assessment was completed on the 26th, 11th, 26th and 10th day, respectively, for CSI, BSC, RSI and SSC. Based on the disease severity scores recorded, the disease incidence (D_I) was obtained by converting all the infected scores to 1 and the healthy scores to 0.

Statistical analysis

By using the GLM procedure of SAS software (SAS INSTITUTE INC., 1992), ANOVAs were carried out to examine the clonal effect and variation in susceptibility traits, *i.e.* disease severity (D_S) and infected incidence (D_I), of eucalypts to bacterial wilt for each inoculation method under the model:

$$Y_{ij} = \mu + C_i + e_{ij}, \quad (1)$$

where Y_{ij} = the observed value, μ = the mean value of all clones tested, C_i = the clone effect, e_{ij} = the residue. The original and transformed values ($\sqrt{Y_{ij}}$) of D_S and D_I were compared in analysis, which turned out the same or similar conclusions. Therefore, we adopted the original score or value for both traits throughout the paper.

For a given trait, the clonal ramet based repeatability (R_R), which is identical to the intra-class correlation (t), and its standard error [$Se(R_R)$ or $Se(t)$] were calculated using the following equations (BECKER, 1992; BALTUNIS and BRAWNER, 2010),

$$R_R = t = \frac{\sigma_C^2}{\sigma_C^2+\sigma_E^2}, \text{ and} \quad (2)$$

$$Se(R_R) = \frac{(1-t)[1+(s-1)t]}{\sqrt{s(s-1)(n-1)/2}}, \quad (3)$$

where σ^2_C = the clone variance component, σ^2_E = the within-clone error, n = the clone number, and s = the number of ramets (*i.e.* cuttings or shoots) per clone. The clonal mean based repeatability (R_C) and standard error [$Se(R_C)$] were estimated as follows (WRIGHT, 1976; ZHANG *et al.,* 2003; ISIK *et al.,* 2005; BALTUNIS and BRAWNER, 2010):

$$R_C = \frac{\sigma_C^2}{\sigma_C^2+\sigma_E^2/s}, \quad (4)$$

$$Se(R_C) = \frac{(1-t)[1+(s-1)t]}{(s-1)\sqrt{s(n-1)/2}}. \quad (5)$$

Obviously, R_R and R_C, as well as their standard errors, are closely related to each other. Both parameters could well represent the clonal or genetic (additive plus non-additive) variation (BORRALHO *et al.,* 1992; GAN *et al.,* 2004). On the other hand, R_C could directly be used to predict genetic gain from clonal selection (BORRALHO *et al.,* 1992; ZHANG *et al.,* 2003; ISIK *et al.,* 2005). In addition, we also calculated the simple correlation among four inoculation methods by use of the least-squared means in D_I or D_S of common clones obtained from ANOVAs (SAS INSTITUTE INC., 1992).

DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

Results and Discussion

Wilt symptom development

Wilting symptom on the infected cuttings or shoots first appeared on the tips of few leaves or shoots and then expanded to the whole (all leaves) cuttings or shoots. As the disease developed, brown and dark discoloration might be observed on the wounds of shoots or roots (WANG *et al.,* 2011). The overall mean infected incidence (D_I) and disease severity (D_S) were plotted against days after pathogen inoculation for each inoculation method, with the significance tests indicated for the corresponding clone effects (*Figure 1*). These results well exhibited the disease development process, expected distinction among inoculation methods, and consistent variation among eucalypt clones tested during the disease development (DIANESE and DRISTIG, 1993; SHI *et al.,* 2000; HÜBERLI *et al.,* 2002; HUANG *et al.,* 2008; RUZ *et al.,* 2008; WANG *et al.,* 2011). They could provide a good opportunity to examine the clonal variation associated with specific inoculation method.

Leaf or shoot drooping was first observed at day 6, 2, 4 and 1 after inoculation by using CSI, BSC, RSI and SSC, respectively. Subsequently, both D_I and D_S increased rapidly for BSC, RSI and SSC, but slowly for CSI. D_I approximately approached maximum at day 16, 7, 18 and 7, and D_S at day 20, 8, 24, and 9, for CSI, BSC, RSI and SSC, respectively, after pathogen inoculation. Obviously, BSC, RSI and SSC had higher D_I and D_S than CSI that was more or less similar to a real field situation, *e.g.* wilt infection in nursery or newly planting sites (LIN *et al.,* 1996; HUANG *et al.,* 2008; LUO *et al.,* 2013). In terms of the infection process norm and period, BSC and SSC were similar while RSI was relatively close to CSI. At the end of assessment, RSI resulted in the highest D_I and D_S (0.60 and 2.4), followed in order

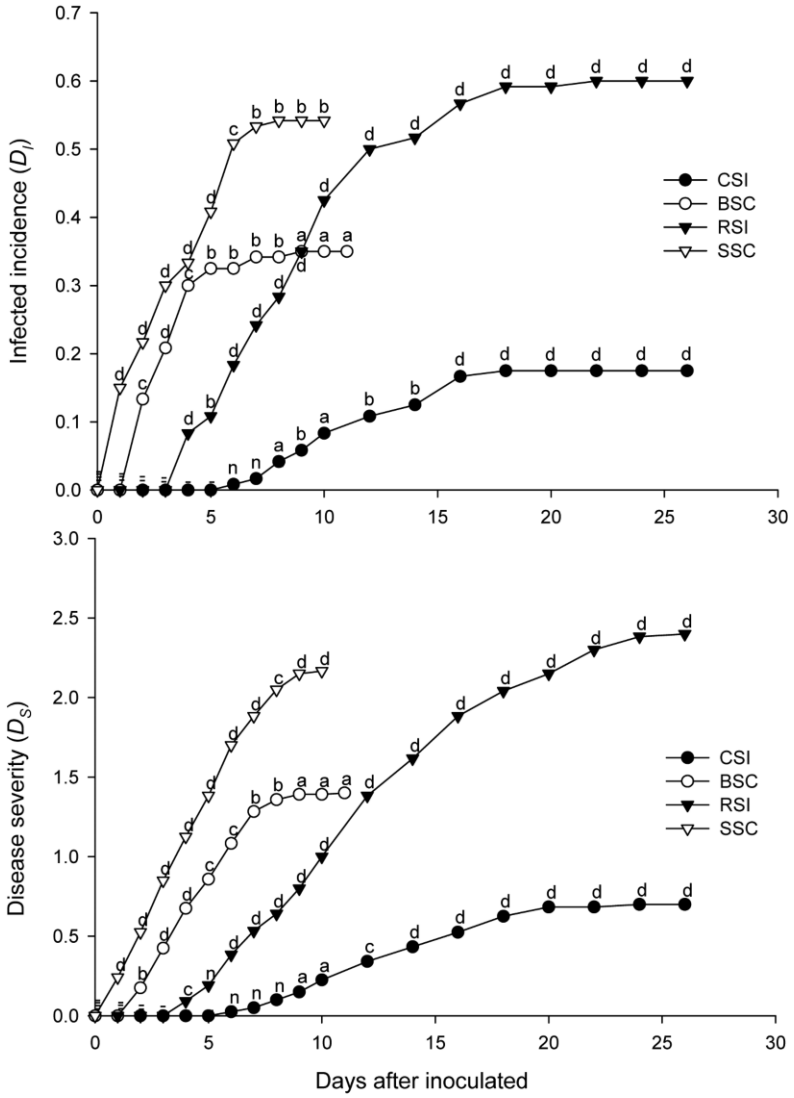

Figure 1. – Average disease infected incidence (D_I) and severity (D_S) against days after inoculation for four inoculation methods. The letters associated with the average values indicated the significance level of the test of the difference among clones: –, not available; n, $P>0.05$; a, $0.01<P\leq0.05$; b, $0.001<P\leq0.01$; c, $0.0001<P\leq0.001$; d, $P\leq0.0001$.

DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

Table 2. – Mean value, clonal mean repeatability and their standard errors (in parenthesis) of the disease infected incidence and severity at final and early assessment with maximum R_C for four inoculation methods.

Inoc method	Early assessment with maximum R_C						Final assessment				
	D_I			D_S			Day	D_I		D_S	
	Day	Mean	R_C	Day	Mean	R_C		Mean	R_C	Mean	R_C
CSI	18	0.18 (0.035)	0.83 (0.047)	24	0.70 (0.139)	0.83 (0.047)	26	0.18 (0.035)	0.83 (0.047)	0.70 (0.139)	0.83 (0.047)
BSC	3	0.21 (0.037)	0.77 (0.047)	3	0.43 (0.085)	0.78 (0.048)	11	0.35 (0.044)	0.51 (0.039)	1.40 (0.175)	0.51 (0.039)
RSI	22	0.60 (0.045)	0.92 (0.038)	20	2.15 (0.170)	0.93 (0.038)	26	0.60 (0.045)	0.92 (0.038)	2.40 (0.180)	0.92 (0.038)
SSC	2	0.22 (0.038)	0.87 (0.046)	3	0.85 (0.129)	0.84 (0.047)	10	0.54 (0.046)	0.62 (0.043)	2.17 (0.183)	0.62 (0.043)

by SSC (0.54 and 2.2), BSC (0.35 and 1.4) and CSI (0.18 and 0.7) (*Figure 1*; *Table 2*).

Clonal variation

Except for no or low infection at the initial stage, the clones tested significantly differed in D_I and D_S no matter which inoculation method was used (*Figure 1*; *Table 3*). The result indicated a high degree of variation among eucalypt clones and/or species tested (Dianese and Dristig, 1993; Old *et al.,* 2003; Gan *et al.,* 2004; Huang *et al.,* 2008). In general, the significance level of the test of the difference in D_I and D_S steadily increased with days after inoculation when using CSI and RSI, but showed a confusing pattern under BSC and SSC. By using BSC, the clones differed in both D_I and D_S with $P<0.001$ at day 2 and $P<0.0001$ at day 3, and then gradually downgraded to $P=0.012$. By using SSC, the clones differed in D_I and D_S with $P<0.0001$ for the first 5 and 7 days respectively, and with $P<0.01$, much weak but still significant, for the rest of assessment.

Clonal ramet based repeatability (R_R) or intra-class correlation (t) measured the fraction of the clonal or genetic variation in the total phenotypic variation (Becker, 1992; Baltunis and Brawner, 2010), which was presented against days after inoculation for each inoculation method in *Figure 2*. R_R in D_I and D_S for CSI and RSI were initially low, rapidly increased within 10 to 14 days, and subsequently reached a stable value till the end of assessment. Changing in another pattern, R_R in D_I and D_S for BSC and SSC increased from a relatively high value at the beginning, reached a peak the next day, and then quickly dropped to values significantly lower than those for CSI and RSI. Difference between BSC and SSC was small. The changing pattern of inoculation methods might imply the complicated role in the infection process of the external conditions including the imposed physiological status (in-vitro culture, tissue injury, vigour etc.) of materials, culture environment and their interaction, besides the genetic aspect of materials.

At the end of assessment, R_R in both D_I and D_S converged at the same value for all inoculation methods because the cuttings or shoots inoculated either survived or died. RSI yielded the highest R_R (0.67 ± 0.085), followed in order by CSI (0.45 ± 0.106), SSC (0.22 ± 0.096) and BSC (0.15 ± 0.088). This ranking was not fully in consistent with that based on D_I or D_S values (*Figure 1*). It seemed that RSI had relatively lower standard error of R_R as well as R_C (*Table 2*) than other inoculation methods. For each inoculation method, a specific early day appeared with the highest R_R in both D_I and D_S (*Figure 2*), which probably better revealed genetic or clonal variation during the disease development. In other word, the genetic or clonal effect might be relatively enhanced or the environment effect lowered at a specific time.

Clonal mean based repeatability

Closely related to the intra-class correlation or clonal ramet based repeatability, the clonal mean based repeatability (R_C) varied in a similar pattern but with higher value over time as indicated in *Figure 2*. In general, R_C ranged from moderate for BSC and SSC to high for CSI and RSI. At the final assessment of four inoculation methods, RSI yielded the highest R_C for both D_I and D_S, followed in order by CSI, SSC and BSC (*Table 2*). In the phenotypic expression of clonal materials, the genetic variance component includes additive and non-additive effects (Borralho et al., 1992; Zhang *et al.,* 2003; Isik *et al.,* 2005). As both additive and non-additive gene actions were suggested important for bacterial wilt resistance (Neto *et al.,* 2002; Gan *et al.,* 2004), the enhanced clonal variation or high clonal mean repeatability could be efficiently utilized to select and deploy resistant clones in practice.

If an early assessment was preferred, CSI and RSI were found roughly at day 18 and 12, respectively, to have high R_C that was the same as or similar to that at the final assessment (referred to *Figure 2*). In contrast, BSC and SSC yielded maximum R_C in D_I and D_S at day

DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

Table 3. – Clone means, overall mean and their standard errors (in parenthesis) of the disease incidence (D_I) and severity (D_S) of infected bacterial wilt at final assessment for four inoculation methods.

Clone	CSI		BSC		RSI		SSC	
	DI	DS	DI	DS	DI	DS	DI	DS
C01	0.83(0.167)	3.33(0.667)	0.50(0.224)	2.00(0.894)	0.83(0.167)	3.33(0.667)	0.67(0.211)	2.67(0.843)
C02	0.00(0.000)	0.00(0.000)	0.33(0.211)	1.33(0.843)	0.00(0.000)	0.00(0.000)	0.50(0.224)	2.00(0.894)
C03	0.17(0.167)	0.67(0.667)	0.50(0.224)	2.00(0.894)	1.00(0.000)	4.00(0.000)	-	-
C04	0.17(0.167)	0.67(0.667)	0.33(0.211)	1.33(0.843)	1.00(0.000)	4.00(0.000)	0.83(0.167)	3.33(0.667)
C05	0.00(0.000)	0.00(0.000)	0.67(0.211)	2.67(0.843)	1.00(0.000)	4.00(0.000)	0.83(0.167)	3.33(0.667)
C06	0.00(0.000)	0.00(0.000)	0.67(0.211)	2.67(0.843)	0.83(0.167)	3.33(0.667)	-	-
C07	0.00(0.000)	0.00(0.000)	0.33(0.211)	1.33(0.843)	1.00(0.000)	4.00(0.000)	-	-
C08	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.50(0.224)	2.00(0.894)
C09	0.00(0.000)	0.00(0.000)	0.33(0.211)	1.33(0.843)	0.50(0.224)	2.00(0.894)	-	-
C10	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	-	-
C11	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.17(0.167)	0.67(0.667)	-	-
C12	0.33(0.211)	1.33(0.843)	0.33(0.211)	1.33(0.843)	0.67(0.211)	2.67(0.843)	-	-
C13	0.33(0.211)	1.33(0.843)	0.50(0.224)	2.00(0.894)	1.00(0.000)	4.00(0.000)	-	-
C14	0.00(0.000)	0.00(0.000)	0.17(0.167)	0.67(0.667)	0.33(0.211)	1.33(0.843)	0.17(0.167)	0.67(0.667)
C15	0.67(0.211)	2.67(0.843)	0.67(0.211)	2.67(0.843)	1.00(0.000)	4.00(0.000)	-	-
C16	0.83(0.167)	3.33(0.667)	0.83(0.167)	3.33(0.667)	1.00(0.000)	4.00(0.000)	0.83(0.167)	3.33(0.667)
C17	0.17(0.167)	0.67(0.667)	0.50(0.224)	2.00(0.894)	0.83(0.167)	3.33(0.667)	-	-
C18	0.00(0.000)	0.00(0.000)	0.33(0.211)	1.33(0.843)	0.83(0.167)	3.33(0.667)	-	-
C19	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	-	-
C20	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.00(0.000)	0.33(0.211)	1.33(0.843)
C21	-	-	-	-	-	-	0.33(0.211)	1.33(0.843)
C22	-	-	-	-	-	-	0.00(0.000)	0.00(0.000)
C23	-	-	-	-	-	-	0.17(0.167)	0.67(0.667)
C24	-	-	-	-	-	-	0.67(0.211)	2.67(0.843)
C25	-	-	-	-	-	-	0.67(0.211)	2.67(0.843)
C26	-	-	-	-	-	-	0.83(0.167)	3.33(0.667)
C27	-	-	-	-	-	-	0.33(0.211)	1.33(0.843)
C28	-	-	-	-	-	-	0.00(0.000)	0.00(0.000)
C29	-	-	-	-	-	-	0.83(0.167)	3.33(0.667)
C30	-	-	-	-	-	-	0.83(0.167)	3.33(0.667)
C31	-	-	-	-	-	-	0.83(0.167)	3.33(0.667)
C32	-	-	-	-	-	-	0.67(0.211)	2.67(0.843)
Mean	0.18(0.035)	0.70(0.139)	0.35(0.044)	1.40(0.175)	0.60(0.045)	2.40(0.180)	0.54(0.046)	2.17(0.183)

2 or 3, which were however much higher than those at the final assessment, greatly narrowing their difference with other two inoculation methods (*Table 2*). Therefore, an efficient inoculation method should simultaneously accelerate the infection process of disease (*Figure 1*) and favour a better and earlier expression of genetic effect (*Figure 2*). The high clonal mean repeatability was achieved through enhanced genetic variance or low environment effect (ERICSSON, 1997).

Although RSI was superior in detecting the disease infection of bacterial wilt and the expression of genetic effect, it seemed that the level of disease infection (D_I and D_S) was not clearly related to the relative genetic variation (R_C). In comparison to other inoculation methods, CSI had a much low D_I and D_S but still high R_C in both traits (*Tables 2* and *3*; *Figures 1* and *2*). Both BSC and SSC had significantly lower D_I and D_S but maximum and much higher R_C at day 2 or 3 than at the final assessment (day 10 and 11). The superiority of RSI might be attributed to the fine control of the environment effect (ERICSSON, 1997) during the puncturing inoculation.

DOI:10.1515/sg-2014-0004
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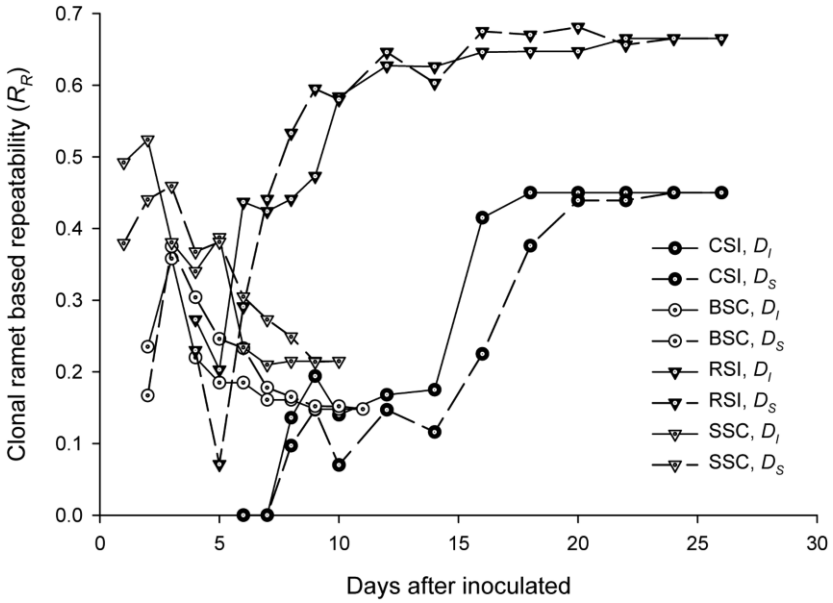

Figure 2. – Clonal variation expressed as clonal ramet based repeatability (R_R) of disease infected incidence (D_I) and severity (D_S) against days after inoculation for four inoculation methods tested.

Table 4. – Correlation coefficients (above diagonal) with significant test (P, below diagonal) among four inoculation methods for the disease infected incidence (D_I) or severity (D_S) at the final assessment (same for both D_I and D_S).

Inoculation Method	CSI	BSC	RSI	SSC
CSI	-	0.596[a]	0.492[a]	0.480[b]
BSC	0.006	-	0.837[a]	0.758[b]
RSI	0.028	<0.0001	-	0.798[b]
SSC	0.229	0.029	0.018	-

[a] $df = 18$; [b] $df = 6$.

Correlation between inoculation methods

Simple correlation between two inoculation methods was essentially the same or equivalent for both disease infection traits (D_I and D_S) at the final assessment. All infected cuttings or shoots finally had either 0 or 1 for D_I, and correspondingly 0 or 4 for D_S. Based on the least-squared means in D_I or D_S of common clones included, the pair correlation was calculated, which was similar to the genetically expressed interaction in the disease infection traits between two inoculation methods or Type-B genetic correlation (BURDON, 1977; JOHNSON, 1997; ZHANG *et al.,* 2003; BALTUNIS and BRAWNER, 2010).

Except for between CSI and SSC, all inoculation methods were significantly positively correlated to each other (*Table 4*). The exception without significant correlation was probably caused by the large error associated with the small sample size, *i.e.* 8 common clones shared by CSI and SSC. BSC had strong relationship with RSI ($P < 0.0001$) and CSI ($P = 0.006$), while other correlations were significant at level of $P < 0.05$. SSC had significant correlation with BSC and RSI at level of $P < 0.05$, probably attributable to the relatively similar physiological status of materials inoculated. Similar relationship among four inoculation methods could be found when the maximum R_C at the early assessment was considered. In contrast to the difference in the disease infection process and clonal variation described above among inoculation methods (*Figures 1* and *2*; *Tables 2* and *3*), the correlation analysis confirmed the similarity, effectiveness and relative efficiency of four inoculation methods in testing the susceptibility or resistance of eucalypts to bacterial wilt, revealing the genotypic expression, and screening potentially resistant clones (SHI *et al.,* 2000; HUANG *et al.,* 2008).

Conclusions

Four inoculation methods investigated highly differed in the disease infection process, incidence and severity,

DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics

and the corresponding clonal variation or clonal mean repeatability. The difference in detecting the clonal variation among inoculation methods were reduced when an efficient early assessment was considered. The difference also indicated the possibility of developing efficient inoculation method with relatively enhanced genetic variation or low environment effect in eucalypt bacterial wilt. This conclusion may be generalized to all quantitative traits in genetic testing where reduced environment effect is expected to relatively increase the genetic variation or the accuracy of breeding value prediction. Four inoculation methods were also significantly correlated, implying that they were all effective in testing the susceptibility or resistance of eucalypts to bacterial wilt, and revealing the genotypic expression of the relevant traits.

Among four inoculation methods, the relative clonal variation in susceptibility of eucalypts to bacterial wilt varied but was consistently significant over the whole infection process of pathogen inoculated. An efficient early assessment could be found to obtain high or maximum clonal variation or clonal mean repeatability. The enhanced clonal variation would benefit the improvement of eucalypts resistance to bacterial wilt. With relatively uniform or controllable inoculation operation, root-collar suspension injection (RSI) yielded the highest clonal variation or clonal mean repeatability with less biased estimates (low standard errors), overwhelmingly superior to other three inoculation methods tested. Nevertheless, further investigation is required to explore other efficient inoculation methods, and to improve the efficiency of existing efficient methods from other aspects such as host materials, pathogen inoculum, environmental conditions, etc.

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DOI:10.1515/sg-2014-0004
edited by Thünen Institute of Forest Genetics