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Reprinted from Canadian Journal of Forest Research

Réimpression de la Revue canadienne de recherche forestière

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Volume 29 • Number 8 • 1999

Pages 1192–1196

Canada

Inoculation methods for selecting *Populus tremuloides* resistant to Hypoxylon canker

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Abstract: Canker expansion and the amount of callus tissue formed were measured monthly on 60 ramets from each of five trembling aspen (*Populus tremuloides* Michx.) clones that had been inoculated in wounds with *Entoleuca mammata* (= *Hypoxylon mammatum* (Wahl.) Mill.) over a 12-month period. At the clone level, the prevalence of nonlethal cankers within clones prior to the study had no correlation with canker expansion with three of the five clones. Greenhouse inoculation of ramets derived from the same five clones resulted in the same resistance rankings as main-stem inoculations in the field. One isolate, Hm-27, produced longer cankers, and less callus developed on all clones compared with trees inoculated with the less aggressive strain, Hm-24. Cankers developed only on clones that were inoculated during the months of April through July with April inoculations resulting in the largest cankers. These results indicate that there is a limited time frame when wounds on aspen are susceptible to infection by artificial inoculation with the pathogen as many wound inoculations neither produced cankers nor wound callus. Comparing inoculation methods, either main-stem inoculations in the field or greenhouse inoculations coupled with the natural canker prevalence could be used to include or exclude clones for use in an aspen breeding program.

Résumé : L'expansion du chancre et la quantité de tissus cicatriciel ont été mesurées mensuellement chez 60 ramets de chacun des cinq clones de peuplier faux-tremble (*Populus tremuloides* Michx.) qui avaient été inoculés artificiellement dans des blessures sur une période de 12 mois avec l'*Entoleuca mammata* (= *Hypoxylon mammatum* (Wahl.) Mill.). À l'échelle des clones, la prévalence de chancres non létaux dans chaque clone antérieurement à cette étude n'était pas corrélée avec l'expansion des chancres chez trois des cinq clones. Les inoculations en serres de ramets provenant des cinq mêmes clones ont donné les mêmes résultats que les inoculations sur le tronc effectuées sur le terrain pour ce qui est du rangement des clones en fonction de leur résistance. Comparativement aux arbres inoculés avec la souche moins agressive Hm-24, l'isolat Hm-27 a provoqué des chancres plus longs et la formation de moins de tissus cicatriciel chez tous les clones. Les chancres se sont développés uniquement sur les clones inoculés durant les mois d'avril à juillet et les inoculations effectuées en avril ont produit les plus gros chancres. Ces résultats montrent qu'il y a une fenêtre de temps limitée, pendant laquelle les blessures sur le peuplier sont sensibles à l'infection suite à l'inoculation artificielle du pathogène, étant donné que plusieurs blessures inoculées n'ont ni produit de chancre ni provoqué la formation de tissus cicatriciel. La comparaison des méthodes d'inoculation, soit les inoculations sur le tronc effectuées sur le terrain ou les inoculations en serres, couplée à la prévalence des chancres d'origine naturelle, pourrait être utilisée pour inclure ou exclure les clones qui devraient faire partie d'un programme d'amélioration du peuplier faux-tremble.

[Traduit par la rédaction]

Introduction

Trembling aspen (*Populus tremuloides* Michx.) is one of the most important hardwood species in the Lake States for pulpwood use, comprising 47% of the total production (4.1×10^6 cords) (Piva 1997). Hypoxylon canker of aspen caused by the fungus *Entoleuca mammata* (= *Hypoxylon mammatum* (Wahl.) Mill.) (Rogers and Ju 1996) is a major cause of aspen mortality (Anderson 1964). Significant differences in the susceptibility of aspen clones to *E. mammata* have been observed in the field (Copony and Barnes 1974), but progress in increasing aspen productivity through selection and breeding has been severely limited as resistant genotypes cannot be identified until they reach 10 years of age (Li et al. 1993).

Previous trials using aspen and this pathogen have included the inoculation of seedlings (Bagga and Smalley 1969), stems (Anderson and French 1972), branches (Griffin et al. 1984), and the exposure of stem tissue or leaves to fungal toxins (Stermer et al. 1984). Data collected from long-term aspen trials indicated that significant differences are present within aspen families (Li and Wyckoff 1991). Although previous tests to screen for resistant aspen had not reliably predicted field performance (Valentine et al. 1976), significant differences were observed in host response in a greenhouse using an artificial inoculation technique to accurately differentiate two open-pollinated aspen families at the seedling stage (Enebak et al. 1996). Histological examination of the inoculated seedlings showed that resistant seedlings produced callus and lignified cells more rapidly and to

Received September 9, 1998. Accepted March 3, 1999.

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a greater extent than susceptible seedlings (Enebak et al. 1997).

A limitation to the screening technique described by Enebak et al. (1996) is the time required to collect root sprouts and scions and propagate them for inoculation. A method to bypass these time-consuming steps would be to screen parental clones in the field. However, some aspen clones support many cankers for long periods of time, while other trees in the clone die rapidly, and yet others remain relatively disease free throughout their life (Anderson and Martin 1981). Griffin et al. (1984) tried to predict resistance by monitoring the amount of wound callus growth and canker elongation by branch inoculation with some success, but others have noted a lack of correlation between canker elongation on inoculated branches and natural infection levels (French and Hart 1978).

In greenhouse tests the time of inoculation affected infection success and canker elongation (Enebak et al. 1996). This temporal effect is not unusual, and similar differences in susceptibility and time of wounding have been observed in other disease systems such as oak wilt (Juzwik and French 1985), chestnut blight (Hebard et al. 1984), peach canker (Biggs 1984), and cherry canker (Bakarat and Johnson 1997). Both wound age and time of branch collection has been reported to affect Hypoxylon canker development (Bagga and Smalley 1969), but little is known concerning the effect of time of wound inoculation on host response in screening trials. The ability to correlate canker expansion and infection on a particular aspen ramet in the field, without having to bring it into the greenhouse would greatly enhance our ability to select resistant aspen. For a rapid screening program to be effective it must reliably rank aspen clones as resistant or susceptible at different times in the life of the host. Therefore, this study was undertaken to evaluate and compare various methods of evaluating aspen clones for resistance using natural infection (prevalence) and artificial inoculations of main-stem inoculations in the field and greenhouse over one growing season.

Materials and methods

Aspen clone delineation

In October 1994, five aspen clones were selected in Itasca County, Minnesota, for a year-long inoculation trial. The clones were located on a well-drained site within 600 m of one another and differed in bark color, texture, branch angle, tree form, and fall coloration. The boundaries of each clone were located and 75 healthy trees within each clone were flagged for future inoculation. At this time all naturally occurring branch and main-stem cankers were tallied. The age of the ramets within the clones ranged from 15 to 22 years.

Field and greenhouse inoculations

Beginning the first week in January 1995 and the first week in every month thereafter, five ramets within each clone were permanently tagged and included in the inoculation trials. On the main stem of each tree at breast height and at the four cardinal directions, four 8-mm diameter bark wounds were made to the cambial layer using a leather punch. Opposite wounds (north and south) received a similar size piece of ME (2% malt extract) agar that contained either Hm-27, a highly aggressive strain of *E. mammata* collected near Appleton, Wis., or Hm-24 a less aggressive strain collected near Grand Rapids, Minn. (Enebak et al. 1996). Alternate (east and west) wounds served as controls and received a similar piece of sterile agar. After wounding and inoculation, Parafilm® was used to wrap all four wounds and duct tape was placed over the laboratory film to maintain the film and agar pieces at the wound. The laboratory film and tape remained on trees until May 1995 for those inoculated in January through April 1995. Trees inoculated in May through September 1995 had the laboratory film and tape removed 1 month after inoculation. The film and tape remained until May 1996 for those ramets inoculated in October through December 1995.

Beginning in February 1995, and every month thereafter, the inoculated trees were examined. For example, the five ramets in each of the clones inoculated in January were examined. In March 1995, 10 ramets were measured: the 5 ramets inoculated in January 1995, and the 5 inoculated in February 1995. This monthly inoculation of new ramets within the clone and measurement of previous inoculations continued through January 1996 resulting in 60 inoculated ramets in each of 5 clones. Canker elongation was measured along the length of the main bole from the center of the wound, while callus formation was scored as (0) no callus formation, (1) slight callus formation on either side of wound, (2) callus formation on at least one side of wound, or (3) complete closure at wound site. In May 1996, the bark was removed from all wound sites to examine the host response at the cambial layer.

Clonally propagated plants were inoculated in a greenhouse to compare clone responses to those in the field. Scions of each clone were collected in February 1995 and grafted onto aspen root stock. Ramets were placed in the greenhouse and watered every 3 days to field capacity. An application of fertilizer (Peters® 20:20:20 (N:P:K), 0.350 g/L) was given once a week. Day and night temperatures in the greenhouse were approximately 22 and 15°C, respectively, and plants were exposed to light intensity of 2.5 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied by high-pressure mercury and sodium lamps on a 18 h light : 6 h dark photoperiod.

In April 1995, 1 × 3 × 3 mm scalpel wounds were made on each of the five clones. At inoculation, seedlings were approximately 3 m tall and 10–15 mm diameter at the soil level. A pair of wounds was made approximately 4 cm apart between the ninth and tenth distal leaves along the stem from the top of the seedling. One of each wound pair received a 4-mm^2 piece of *E. mammata* mycelium, isolate Hm-27, on ME agar. The remaining wound received a similar size piece of sterile ME agar. Wounds were covered with Parafilm® to keep either the fungus or agar in place and to retard desiccation. Both wounds were monitored for canker development weekly for 12 weeks. The inoculation experiment was conducted on 10 ramets per clone and was repeated once.

Analysis of host response and canker development

The means were analyzed as a completely randomized block design and analysis of variance (ANOVA) were made for canker length and callus formation to determine the effects of clone, fungal isolate, and their interactions using SAS version 6.10 (SAS Institute Inc., Cary, N.C.). Month of wounding, stem diameter, wound position, and prevalence of cankers within the clone prior to the experiment were among the host response variables examined from field inoculations. Greenhouse inoculations examined the effects of clone and fungus on canker length and callus formation.

Results

Field inoculations

Canker initiation and elongation after wound inoculation differed by clone, month of wounding, and isolate used (Table 1). While, significant differences were observed for clone by month interactions, there was no diameter nor

Table 1. Analysis of variance of canker elongation and callus formation on aspen artificially inoculated over a 12-month period with *Entoluca mammata* in Itasca County, Minnesota.

	Canker length				Callus formation			
Source	df	MS	*F*	*P*	MS	df	*F*	*P*
Clone (C)	4	5 734.68	21.77	0.0001	19.74	4	19.74	0.0001
Month (M)	10	29 559.77	112.22	0.0001	197.30	10	197.30	0.0001
Isolate (I)	2	163 712.46	621.51	0.0001	442.98	2	442.98	0.0001
Diameter (D)	11	2 669.10	10.13	0.1251	14.15	11	14.15	0.2851
Canker age (A)	18	2 476.37	9.40	0.0001	177.17	18	177.17	0.0001
C × M	40	2 910.80	11.05	0.0001	10.42	40	10.42	0.0001
C × I	8	4 958.22	18.82	0.3451	4.42	8	4.42	0.3871
C × D	28	1 087.23	4.13	0.0701	5.08	28	5.08	0.1021
C × A	68	493.03	1.87	0.0001	1.04	68	1.04	0.0001
Error	8555	263.41		0.0001		8555		0.0001

Table 2. Characteristics of aspen clones used in the year-long inoculation trial in Itasca County, Minnesota and their resistance rankings pre- and post-inoculation with *Entoluca mammata* strain Hm-27.

	Clones prior to inoculation					Clones after field inoculations			Clones after greenhouse inoculations			
Clone	Area (ha)	Members	Cankers*	Percent	Ranking	Length (cm)	Callus	Ranking	No.	Percent†	Length (cm)	Ranking
Alpha	0.08	289	30	10.3	3	22.5*a*	1.3*a*	5	10	90	13.5*a*	5
Beta	0.04	170	21	12.3	4	19.6*b*	1.2*a*	4	11	54	9.3*ab*	4
Gamma	0.02	173	29	16.7	5	14.2*d*	1.1*b*	1	11	9	1.3*c*	1
Delta	0.06	367	26	7.0	2	15.2*d*	1.1*b*	2	12	16	1.5*c*	2
Epsilon	0.09	475	24	5.0	1	17.6*c*	1.0*b*	3	10	30	5.0*b*	3

Note: Ranking of clones prior to inoculation were based on the prevalence of cankers, which indicated a putative decreasing tolerance to *E. mammata*. Rankings of clones after field and greenhouse inoculations were based on canker expansion and indicated a decreasing ability to withstand infection and canker development. Treatment means followed by the same letter within a column do not differ significantly ($p = 0.05$) according to Duncan's mean separation test.

*Number of trees with naturally occurring *E. mammata* cankers in December 1995.

†Percentage of ramets on which cankers developed after inoculation.

Table 3. Expansion of main stem cankers on aspen inoculated with *Entoluca mammata* over a 12-month period in Itasca County, Minnesota in 1995.

	Canker expansion (cm)												
Clone	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	SE*
Alpha	0	0.2	0.6	0*b*	1.3*a*	7.5*a*	0.4	0	0	0	0	0	1.8
Beta	0	0	0.9	1.4*a*	2.8*a*	4.1*b*	0	0.6	0	0	0	0	2.1
Gamma	0	0	0.4	0.2*b*	0.8*b*	2.2*c*	0	0	0	0	0	0	1.3
Delta	0	0	0.2	0*b*	0.3*b*	1.4*c*	0.6	0.9	0	0	0	0	1.6
Epsilon	0	0	0.2	0.3*b*	1.6*a*	4.6*b*	1.2	0	0	0	0	0	1.1

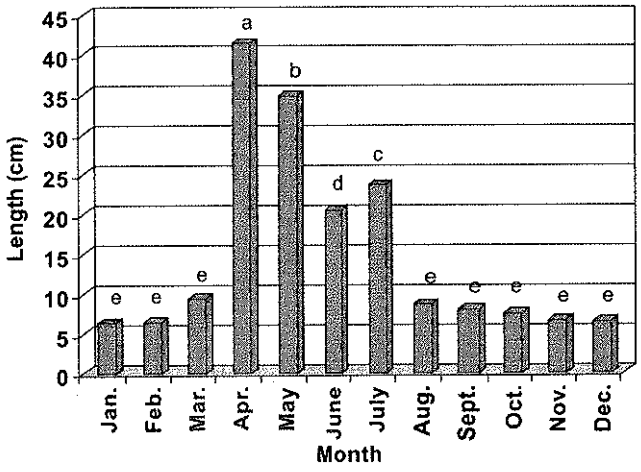
Note: Treatment means followed the same letter within a column do not differ significantly ($p = 0.05$) according to Duncan's mean separation test. Columns without letters indicate no differences between clone and canker expansion.

*Values are the SE within a clone across the 12-month experiment.

clone by fungus interactions for either canker length or callus (Table 1). The average canker length for all inoculations at the end of the 18 months ranged from 22.5 to 14.2 cm for the five clones (Table 2). Artificial inoculation with *E. mammata* resulted in clones Alpha, Beta, and Epsilon with the longest cankers that expanded rapidly during the months of April, May, June, and July (Table 3). Cankers on Delta developed significantly slower and later in the season (Table 3). Cankers developed only on trees that had been inoculated during the months of April through July 1995 (Fig. 1). Within the growing season, the greatest canker elongation occurred during June (7.5 cm) and was greatest for clones Alpha, Epsilon, and Beta, followed by Gamma and Delta, respectively (Table 3).

Callus production (wound closure) on inoculated and control wounds varied by clone and date of wounding (Table 2). The greatest amount of wound callus formation occurred on Alpha (1.3), the least on Epsilon (1.0), and the other three clones were intermediate. Trees wounded in January through April did not produce callus until May 1995 but, at the end of the experiment, had produced more callus than trees wounded made any month thereafter. Trees wounded after July 1995, however, did not produce callus during the rest of the experiment, and wounds remained open. Canker length

Fig. 1. Canker length by month of inoculation caused by *E. mammata* on five aspen clones. Means represent 10 wounds made on each of 5 clones measured at the end of the 18-month experiment. Bars with different letters are significantly different ($p = 0.05$) according to Duncan's mean separation test.



and callus formation by month for all clones measured with the bark intact was not different from measurements made with the bark removed after 18 months as fungal invasion and cambial mortality had not proceeded more than 7 mm beyond what was observed on the bark surface.

Greenhouse inoculations

Canker initiation and expansion of inoculated and control wounds on greenhouse grown ramets are presented in Table 2. Canker lengths after 12 weeks ranged from 1.3 to 13.5 mm on the 10–12 ramets of each clone inoculated including noninfected stems. The largest (13.5 mm) and the most cankers (90%) developed on ramets derived from the Alpha clone and the smallest and the least on the Delta clone. Relative susceptibility rankings based on canker expansion in the greenhouse was similar to that observed on field-inoculated clones (Table 2).

Discussion

Canker length and callus formation in response to *E. mammata* inoculation varied significantly with respect to clone and the time of inoculation. Based on the prevalence of cankers in the field prior to inoculation, we ranked Gamma to be the most resistant to lethal cankers, as individual ramets within the clone supported more cankers. In contrast, Epsilon, with the fewest number of cankered trees, was ranked as the least tolerant as it apparently did not support cankers (Table 2). When these clones were challenged in the field using main-stem inoculations, cankers on Gamma and Delta were significantly smaller than the most susceptible clones (Alpha and Beta), which had the greatest canker expansion. While there was a shift in rankings with respect to Epsilon and Alpha when comparing prevalence with artificial inoculations, the relative rankings of the five clones tested were similar regardless of field or greenhouse inoculations.

Comparison of different inoculation methods (field and greenhouse) for either inclusion or exclusion of aspen clones from a breeding program has obvious trade-offs. While the propagation and maintenance of aspen in the greenhouse can be completed year round, this process is time consuming and costly, making field inoculations more attractive. However, the lack of correlation between the prevalence of cankers in the field and inoculations with respect to Epsilon and Alpha in this study as well as clones in other studies is common (French and Hart 1978). The discrepancy between what is observed in nature and how the clones perform when artificially challenged with the pathogen could be due to escapes and has made selection of resistant clones based on natural canker prevalence unreliable. While others have noted differences between infection and canker expansion after inoculation (Bruck and Manion 1980), the clones used in this trial were chosen because they were close to one another (within 600 m) and thus minimized the interacting environmental factors that have been reported to influence canker expansion in trials that had clones separated by hundreds of miles (Bagga and Smalley 1969). Thus, the ability of the clones to withstand naturally occurring cankers (prevalence) and to retard canker expansion when artificially inoculated is tied closely to the genotype of the particular clone.

An important aspect of screening aspen is determining the wounding site and timing of artificial inoculations. Previous research on screening aspen for resistance have included the inoculations of branches (French and Hart 1978), seedlings (Bagga and Smalley 1974), or stems (Anderson and French 1972). We inoculated main stems to mimic a natural potentially lethal main-stem infection as well as to duplicate the greenhouse inoculations previously used (Enebak et al. 1996). More importantly, we examined the timing of the inoculations as differences have been noted in the time of inoculation and canker expansion on aspen (Shea 1963; French and Hart 1978) as well as with other pathogens (Biggs 1984; Bakarat and Johnson 1997). The relative rate of infection and canker elongation during the dormant season has not been reported for *E. mammata*. Inoculations by French and Hart (1978) resulted in the greatest canker elongations when made in June and would suggest that the summer months are more appropriate for screening tests. Inoculations made in our trials, beginning in January and ending in December, revealed a temporal effect on canker elongation and wound callus formation, suggesting that *P. tremuloides* has a limited time period when wounds are susceptible to infection. Data indicates that early canker expansion does not necessarily suggest a more susceptible clone than one that begins canker elongation later in the season. These studies clearly demonstrate that the time of field inoculations will affect canker formation and thus a tree's ability to withstand infection by *E. mammata*. Future in situ inoculations in the Lake States region should be conducted no sooner than April and before July, as inoculations made either too early or too late in the season may give spurious results with respect to canker elongation and clone rankings.

Wound closure in aspen may slow the colonization of *E. mammata* after infection and is an important aspect in selecting resistant trees (Griffin et al. 1984; Bucciarelli 1996; Enebak et al. 1997). The majority of callus was formed during May through July, and wound closure during this time is related to the period of greatest tree growth. Clones that had the smallest cankers had a corresponding increase in the

amount of callus produced. However, trees wounded late in the season did not form wound callus or initiate canker expansion even through the next growing season. Thus, the direct relationship between callus and canker expansion is not clear as one would expect.

One of the pitfalls in examining aspen for resistance is that the naturally occurring infection levels recorded at the beginning of this study was just a snapshot in time, and the true canker incidence is unknown. In this study, as well as others that have examined canker prevalence, there is no record of infection rates and mortality over time; both will change with insect and weather activity, which can alter infection and canker expansion within the stand. The prevalence of infection within the clones at the time of study initiation neither reflected nor predicted their ability to tolerate canker expansion after artificial inoculations and canker expansion. Therefore, selection of aspen based on prevalence alone is not an effective method to screen aspen for resistance nor is early or late season inoculations, as these failed to produce any cankers at all. Selection strategies that include prevalence rankings as an initial step in selecting aspen clones, which is then coupled with either field or greenhouse inoculations, will result in selecting aspen that may have resistance with a higher level of confidence. Using the inoculation and selection strategies outlined in these studies, clones Gamma and Delta were identified as being tolerant of *E. mammata* and would be useful in aspen breeding programs.

Acknowledgment

The authors appreciate the technical support provided by Mr. Dave Gray of the College of Natural Resources, University of Minnesota.

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