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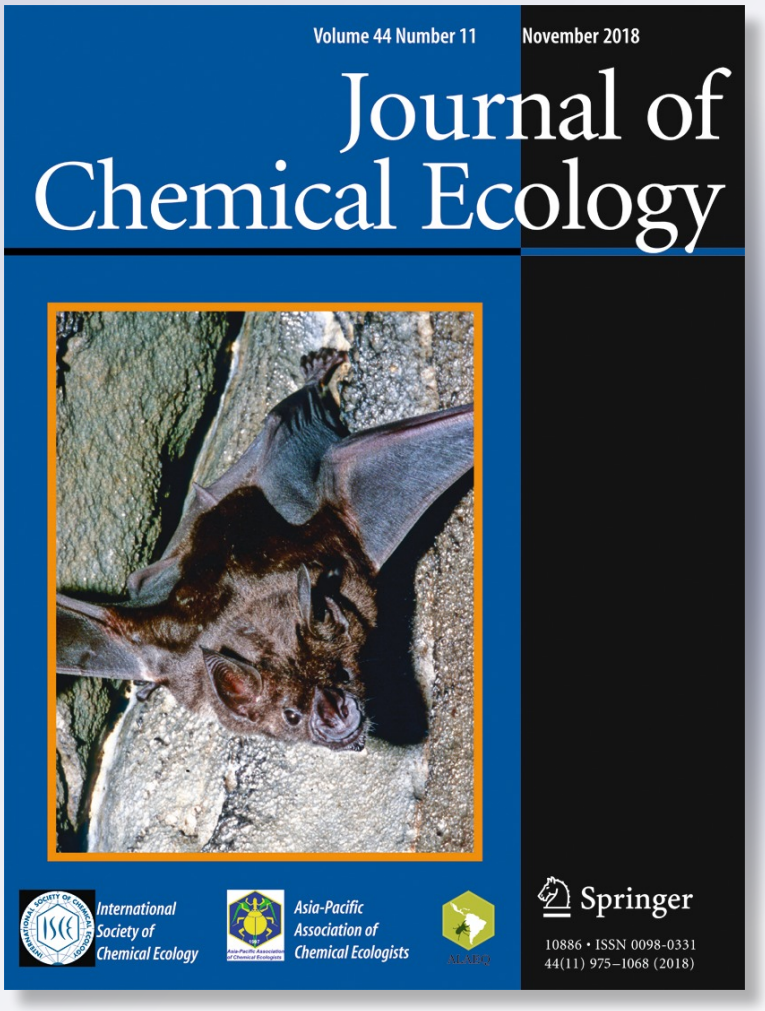


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# Clonal Saplings of Trembling Aspen Do Not Coordinate Defense Induction

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## Abstract

Induction of plant chemical defenses in response to insect feeding may be localized to the site of damage or expressed systemically, mediated by signal transduction throughout the plant. Such systemic induction processes have been widely investigated in plants with single stems, but rarely in clonal plants comprised of multiple ramets with vascular connections. For a clonal tree species such as trembling aspen (*Populus tremuloides* Michx), integration of induced defense within clones could be adaptive, as clones are spatially extensive and susceptible to outbreak herbivores. We used pairs of aspen saplings with shared roots, replicated from three genotypes, to determine whether defense-induction signals are communicated within clones. One ramet in each pair was subjected to a damage treatment (feeding by *Lymantria dispar*, followed by mechanical damage), and subsequent changes in leaf defensive chemistry were measured in both ramets. Responses to damage varied by defense type: condensed tannins (CTs) increased in damaged ramets but not in connected undamaged ramets, whereas salicinoid phenolic glycosides (SPGs) were not induced in any ramets. Genotypes varied in their levels of CTs, but not in their levels of SPGs, and responded similarly to damage treatment. These results suggest that, even with both vascular and volatile information available, young aspen ramets do not induce defenses based on signals or metabolites from other ramets. Thus, unlike other clonal plant species, aspen do not appear to coordinate defense induction within clones. Lack of coordinated early induction in aspen may be related to the function of CTs in tolerance, rather than resistance.

**Keywords** Clonal plants · Condensed tannins · Induction · Phenolic glycosides · *Populus tremuloides* · Gypsy moth · *Lymantria dispar*

## Introduction

Defoliation-induced production of plant chemical defenses occurs across various spatial scales. Local defense induction occurs within the damaged leaf, a scale at which allocation costs are low and damage cues are reliable predictors of future herbivory (Karban et al. 1999). Local induction does, however, leave other parts of the plant undefended and vulnerable to attack. Whole-plant systemic induction is potentially costlier than local induction but can benefit plants by restricting the spread of herbivores and preventing further damage (Heil and Ton 2008). Systemic induced responses can be mediated by within-plant vascular signaling and resource transport (Arnold et al. 2004; Orians 2005), as well as within-plant volatile signaling (Frost et al. 2007; Heil and Ton 2008; Li and Blande 2017).

Vascular connections within clonal plants provide for the transfer of materials among individual ramets. Most research on clonal integration has focused on the sharing of mineral nutrients and photosynthates (Liu et al. 2016). Clonal vascular connections also have the potential to transmit non-nutritive substances – such as defense compounds, precursors thereof, or defense induction signals – using the same mechanisms as does within-plant vascular transport (Stuefer et al. 2004). For example, defense induction signals have been shown to propagate through vascular connections in clonal species of clover and sedge (Gómez and Stuefer 2006; Chen et al. 2011). This integration of defense induction across individual clones has been proposed as a strategy that spreads damage risk across a clonal plant by equalizing within-clone variation in susceptibility and dispersing

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herbivores away from the most vulnerable ramets (Gómez et al. 2008). Because clonal ramets are genetically identical and in close proximity to each other, signals of damage received through clonal connections should be very reliable indicators of immediate herbivory risk. Clonal defense induction is likely constrained by the same source-sink and directional-flow dynamics as clonal resource sharing (Stuefer et al. 2004). However, clonal induction of plant defense has rarely been explored, despite the prevalence of clonal habits among angiosperms (Van Groenendael et al. 1996).

In the clonal tree species trembling aspen (*Populus tremuloides*), within-ramet systemic induction can occur for both of the principal classes of defense compounds – condensed tannins (CTs) and salicinoid phenolic glycosides (SPGs; (Rubert-Nason et al. 2015). Induction is observed most commonly, and at greater magnitude, for CTs relative to SPGs (Rubert-Nason et al. 2015). Defoliation increases carbon transport to aspen roots (Babst et al. 2008), but whether defense signals or compounds themselves are transported into shared roots and transmitted to other connected ramets is unknown. One greenhouse study showed no change in gene expression in undamaged aspen ramets with root connections to damaged ramets (Jelínková et al. 2012). In contrast, a field study demonstrated physiological responses (e.g. increased SLA) of undamaged aspen ramets to defoliation of their connected neighbors (Baret and DesRochers 2011). Both studies used mechanical damage as their defoliation treatment, and neither measured induction of defense compounds, so the question of whether aspen ramets coordinate their chemical responses to herbivory has yet to be addressed. In addition, despite striking genetic variability in both constitutive and induced aspen defenses (Rubert-Nason et al. 2015; Stevens and Lindroth 2005) there is a lack of evidence for whether genetic variation in within-ramet induction translates to genetic variation in the degree of induction communication or coordination between ramets.

Aspen ramets are physiologically integrated through shared root systems (Tew et al. 1969). Integration of defense induction within clones would likely be adaptive in aspen for several reasons. First, aspen is susceptible to defoliation by outbreak herbivores such as *Malocosoma disstria* and *Lymantria dispar,* which can cause widespread damage at high densities. In addition, individual clones can be large in size, so some ramets may have information about an incipient herbivore outbreak before others. Aspen's chemical defenses are costly to produce (Cole et al. 2016; Osier and Lindroth 2006), so the improved accuracy of information about herbivore threats afforded by clonal defense integration could prevent unnecessary investment of limited plant resources.

The purpose if this research was to investigate potential clonal integration of induced defense responses in aspen. We predicted that in clonal aspen ramets with shared root connections, (1) defoliation would cause induction of defense compounds in both damaged ramets and in connected, undamaged ramets, and (2) variation among genotypes in the magnitude of defense induction in damaged ramets would correspond to that in connected undamaged ramets.

## Methods and Materials

The experimental design for this study consisted of a 2 (damage treatment) × 2 (root severing) × 3 (genotype) factorial (four replicates per treatment combination). Each experimental unit was a pair of aspen ramets connected by a shared root. The damage treatment (+/-) was applied to one or none of the ramets in the pair. To preclude potential confusion about treatment nomenclature, we will use the term "damage" to refer only to the defoliation treatment, and the term "severed" to refer to the root severing treatment.

If induction occurs in the undamaged partners of damaged ramets, it could be mediated via either the shared root system or volatile signals. To provide insight into the mechanism of clonal induction, we incorporated a root severing treatment in which root connections were severed midway between paired ramets prior to application of the damage treatment. We also planted undamaged, control pairs of ramets, interspersed among pairs with one damaged ramet, to further control for volatile-mediated defense induction. Induction in the undamaged members of connected ramet pairs, but not in the undamaged members of severed pairs (or in members of undamaged pairs) would confirm signaling via the root pathway. Induction in any undamaged ramets not connected to damaged ramets, on the other hand, would indicate volatile-mediated induction. Because we found no significant induction in undamaged ramets connected to damaged ramets (Results), nor in any undamaged control ramets, data collected from volatile-induction controls are unnecessary and will not be presented.

Root material for this study was collected at Pine Island State Wildlife Area (Portage, Wisconsin, U.S.A.). Each of the three genotypes used was spatially distinct from other clones. We followed root connections when excavating to ensure that all replicate root lengths per clone derived from the same genotype. Collected roots were cut into 100–140 cm lengths, soaked for 1 min in 10% commercial bleach, and rinsed with water before planting. All roots were planted in outdoor raised beds in April 2016, with randomized genotype order. The planting medium was 60% torpedo sand and 40% field top soil (Keleney Topsoil, Madison, Wisconsin, U.S.A.) with no additional nutrient treatment. Roots were planted 3 cm deep and allowed to sprout. When new shoots were several centimeters in height, they were thinned to two ramets, at least 30 cm apart, per root section. The ramets were watered as needed and allowed to grow outside for one year, attaining heights of 0.5–2 m, before the damage treatment was applied.

For ramets receiving the damage treatment, third-instar *Lymantria dispar* larvae were applied for 13 days beginning May 9, 2017. Larvae were acquired from the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (Buzzards Bay, Massachusetts, U.S.A.) as second instars, reared in the lab on a wheat germ and casein-based artificial diet to the third instar, then enclosed in mesh bags on the damage-treatment ramets. To minimize the potential confounding effects of mesh bags on foliar chemistry, all ramets were completely enclosed in mesh throughout the 13-day feeding period. To produce a uniform damage treatment across ramets of different sizes, we adjusted the number of larvae placed on a tree according to tree size. We first sorted the experimental ramets into three height classes. Ramets in the short, medium, and tall height categories received 12, 16, and 20 larvae, respectively. After 13 days of feeding, we removed the insects. We then clipped the damaged ramets to a uniform leaf area loss (~15% removal on every third leaf) with pinking shears, to further standardize the damage treatment.

Leaf samples from all ramets were collected at each of three time points: (1) immediately prior to damage treatment, (2) five days after damage treatment, and (3) three months after damage treatment. Initial leaf flush occurred at approximately the same time across all the experimental trees, so all leaves within Collections 1 and 2 were of similar age. For Collection 3, we restricted our sampling to leaves that had flushed and matured following completion of the damage treatment. For each collection, leaves were harvested haphazardly throughout the canopy. Leaves were removed at the petiole using sharp scissors, which prevents chemical induction due to the collection itself (Mattson and Palmer 1988). Once harvested, leaf samples were vacuum dried and ball-mill ground. CTs were analyzed using the acid butanol method of (Porter et al. 1985), modified as in (Rubert-Nason et al. 2017), and with standardization against tannins purified from *P. tremuloides* as recommended by Hagerman and Butler (1989). SPGs (salicortin, salicin, tremulacin, and tremuloidin) were extracted into methanol and analyzed using ultra-high performance liquid chromatography-mass spectrometry as described by (Rubert-Nason et al. 2017).

We recognize that aspen produces numerous other secondary metabolites such as hydroxycinnamate derivatives (Tsai et al. 2006) and inducible protease inhibitors (Rubert-Nason et al. 2015). However, to date, none of those has been demonstrated to function as an herbivore defense agent in aspen, so they were not addressed in this study.

Statistical analyses consisted of linear models of ramet phytochemistry, with damage treatment, collection time point, and genotype as predictor variables. We also created linear models of the differences in leaf chemistry between connected ramets to confirm whether damage affected pairwise defense coordination. Type III analysis of variance was employed for all models after confirming that assumptions of normality and homogeneity of variance were met.

## Results

In all three genotypes, herbivore damage caused significant induction of CTs in damaged ramets but not in undamaged ramets connected to them (Fig. 1). Levels of CTs were genetically variable: genotypes ranged from 4 to 7% dry weight in mean constitutive (Collection 1) CT concentration. Damaged ramets exhibited proportional increases in CT concentration of 50–100% five days after treatment (Collection 2). The three genotypes responded similarly to damage, so genotype x damage interactions were not included in the final statistical model. Late-season (Collection 3) leaves of damaged ramets, produced after the defoliation event, had CT levels that were moderately elevated but still lower than those of leaves collected soon after damage.

If undamaged ramets had induced their defenses in coordination with connected damaged ramets, differences in CT levels between paired ramets would have remained constant across leaf collections. However, differences in CT levels between paired ramets all increased after damage treatment ($\Delta(CT_{damaged} - CT_{undamaged}) > 0$, Fig. 2), indicating that damaged ramets had defense induction responses that their undamaged counterparts failed to match. Genotypes did not significantly vary in the change in inter-ramet CT difference after damage treatment.

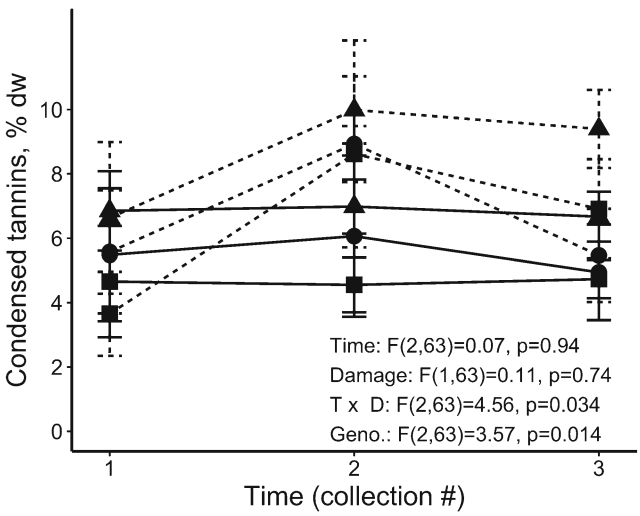


**Fig. 1** Concentrations of foliar condensed tannins (% dry weight) in damaged (dashed lines) and undamaged (solid lines) aspen ramets in root-connected pairs. CTs were measured in leaves collected (1) immediately before, (2) five days after, and (3) three months after damage treatment. Symbols are genotype means and denote the three experimental genotypes. Error bars represent ± 1 SE

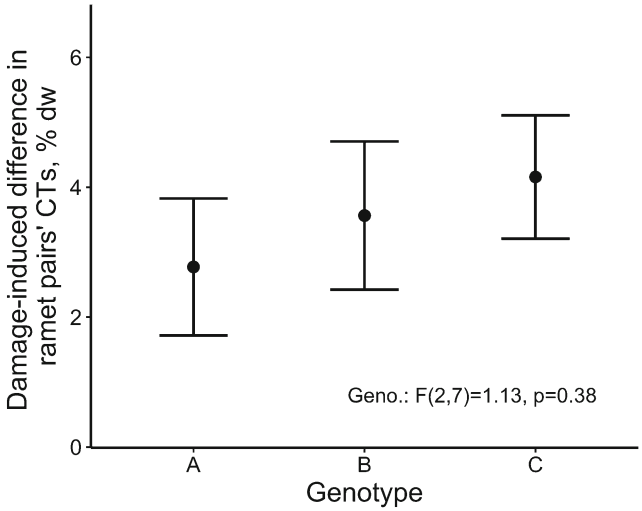


**Fig. 2** Change, from Collection 1 to Collection 2, in *differences* in condensed tannin levels between pairs of connected ramets, for each experimental genotype. Differences in foliar condensed tannins (% dry weight) were calculated as $CT_{damaged} - CT_{undamaged}$ for each leaf collection. Error bars represent ± 1 SE; none intersects or falls below zero

SPG concentrations did not respond to damage treatment (Fig. 3). Levels averaged 7–10% dry weight at Collections 1–2, then increased to 12–14% dry weight at Collection 3. This temporal change was most likely a consequence of leaf age, since the final harvest was of relatively young (but fully-expanded) indeterminate growth. SPG concentrations did not vary significantly among genotypes. Because all genotypes responded similarly to damage, the genotype x damage interaction term was removed from the final statistical model.

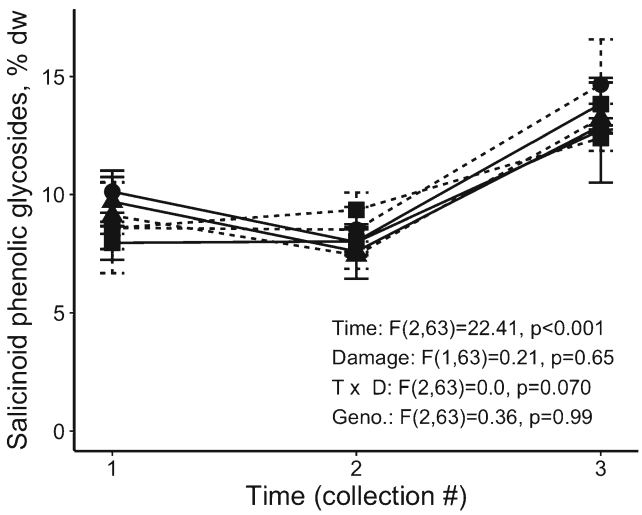


**Fig. 3** Concentrations of foliar salicinoid phenolic glycosides (% dry weight) in damaged (dashed lines) and undamaged (solid lines) aspen ramets in root-connected pairs. SPGs were measured in leaves collected (1) immediately before, (2) five days after, and (3) three months after damage treatment. Symbols are genotype means and denote the three experimental genotypes. Error bars represent ± 1 SE

## Discussion

Herbivore damage caused induction of CTs in trembling aspen ramets but not in the undamaged ramets connected to them. These results suggest that there was no transmission of induced secondary metabolites, their metabolic precursors, or defense induction signals via vascular root connections. Even with both vascular and volatile transmission pathways available, there was no coordination of defense induction between clonally-connected ramets. The total lack of induction coordination was surprising because the ramets in this study were in close proximity, and thus at a spatial scale where clonal communication should be especially feasible and beneficial (Stuefer et al. 2004). Our finding of no clonal coordination of defense induction matches the gene-expression results of Jelínková et al. (2012), and suggests that clonal aspen do not induce defenses based on signals or metabolites from outside the individual ramet.

Our study findings are relevant only to young, even-aged ramets of clonal aspen (as occur after disturbance) and thus may not reflect the coordination abilities of clones with older or uneven-aged ramets. Source-sink dynamics and directionality of root flow have been suggested as constraints that might prevent clonal defense coordination in connected ramets of divergent age (Jelínková et al. 2012; Stuefer et al. 2004). However, root flow in clonal aspen may be bi-directional (Bretfeld et al. 2017), and any limitations arising from source-sink dynamics do not easily explain our results in even-aged ramets. The magnitude of within-ramet defense induction in aspen is context-dependent (Rubert-Nason et al. 2015); if defense coordination between ramets is similarly context-dependent, it may occur in environments different from the one in which this study was performed. Finally, while this study focused on clonal integration of defense induction, systemic priming – preparation for defense without actual induction – has also been shown in *Populus* (Frost et al. 2007) and could potentially extend between ramets. Potential clonal integration of priming is an interesting topic for future research.

If the observed results are generally applicable to aspen, it is possible that early defense induction in response to chemical inputs from neighboring ramets has not evolved simply because it is not beneficial, given the species' chemical and life history traits. For example, clonal coordination might not be adaptive if signals are not fast enough (relative to the timeframe of herbivory) to provide a true early warning (Gómez et al. 2010). For this reason, larger and more widely-spaced plants such as trees may not benefit as much from defense coordination as do small and closely-spaced herbaceous plants. In addition, SPGs are the most generally important insect resistance factors in aspen (Lindroth and St. Clair 2013), and those compounds exhibit only minimal local and systemic induction (Rubert-Nason et al. 2015). So in this respect, absence of inter-ramet coordinated induction is not surprising.

In the specific case of CT induction, trees may not benefit from accelerated induction because this particular class of compound may function more to enhance recovery, rather than protect, from herbivory. Foliar CTs are generally ineffective defenses against common defoliating Lepidoptera, such as those used in this study (although they can reduce damage by aphids and defoliating beetles; (Lindroth and St. Clair 2013; Rubert-Nason et al. 2017). Rather, CT induction may facilitate post-defoliation nitrogen uptake and growth recovery by aspen trees (Madritch and Lindroth 2015). Tannins may thus function as tolerance, not resistance, compounds, which would obviate the need for early induction of tannins in anticipation of future damage. The adaptive value of clonal defense induction may thus depend on the timing of particular defense benefits relative to herbivore damage.

Overall, the induction responses we observed were consistent with previous studies of defense induction in aspen, which have typically reported CT induction of ~3–10% dry weight and SPG induction of only ~0–2% dry weight (Osier and Lindroth 2001; Rubert-Nason et al. 2015; Stevens and Lindroth 2005). Surprisingly, genotypic variation was significant only for CTs, not for SPGs. In past studies, SPGs were the more strongly genetically-determined compounds (Donaldson and Lindroth 2007, 2008; Osier and Lindroth 2006). However, the number of genotypes used in this study was low relative to other studies, so we may have simply not captured enough of the range of intraspecific variation in SPGs to observe a significant genotypic effect. Contrary to our initial predictions, genetic variation in CT induction responses in damaged ramets did not translate to genetic variation in the presence or magnitude of induction responses in undamaged ramets.

In conclusion, this study suggests that young trembling aspen do not use vascular connections to coordinate their defense induction responses within clones. Although aspen exhibit systemic induced responses to herbivore damage at the within-ramet level, that ability does not translate to among-ramet responses or genetic variation therein. The lack of defense induction coordination within aspen clones could contribute to spatial variation in foliar chemistry and the ecological processes that it mediates.

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