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RESEARCH ARTICLE

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What determines root-sprouting ability: Injury or phytohormones?

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Abstract

Premise: Root-sprouting (RS) is an evolutionarily independent alternative to axillary stem branching for a plant to attain its architecture. Root-sprouting plants are better adapted to disturbance than non-RS plants, and their vigor is frequently boosted by biomass removal. Nevertheless, RS plants are rarer than plants that are not root-sprouters, possibly because they must overcome developmental barriers such as intrinsic phytohormonal balance or because RS ability is conditioned by injury to the plant body. The objective of this study was to identify whether phytohormones or injury enable RS.

Methods: In a greenhouse experiment, growth variables, root respiration, and phytohormones were analyzed in two closely related clonal herbs that differ in RS ability (spontaneously RS *Inula britannica* and rhizomatous non-RS *I. salicina*) with and without severe biomass removal.

Results: As previously reported, *I. britannica* is a root-sprouter, but injury did not boost its RS ability. Root respiration did not differ between the two species and decreased continuously with time irrespectively of injury, but their phytohormone profiles differed significantly. In RS species, the auxins-to-cytokinins ratio was low, and injury further decreased it.

Conclusions: This first attempt to test drivers behind different plant growth forms suggests that intrinsic phytohormone regulation, especially the auxins-to-cytokinins ratio, might be behind RS ability. Injury, causing a phytohormonal imbalance, seems to be less important in spontaneously RS species than expected for RS species in general.

KEYWORDS

Asteraceae, auxin/cytokinin ratio, biomass, buds, disturbance, *Inula britannica*, *Inula salicina*, phytohormones, root respiration

Plants have intriguing growth plans that govern their typical architecture (Hallé et al., 1978; Barthélémy and Caraglio, 2007; Teichmann and Muhr, 2015). The first step for building a plant body is the establishment of a primary apical meristem, that originates in the shoot pole of an embryo and produces the primary shoot of a plant (West and Harada, 1993). Further plant development and branching are dependent on the subsequent formation of shoot meristems and their growth. Shoot meristems have two possible origins, either in axillary buds on a stem or in adventitious buds usually on a root (rarely on a leaf or stem internode) (Groff and Kaplan, 1988; Kerstetter and Hake, 1997; Klimešová, 2021). These two origins of the apical meristem are not evenly represented in the flora, have different ecological properties, and are evolutionarily independent, and their differences have received little research interest thus far (Klimešová et al., 2017, 2021; Herben and Klimešová, 2020).

The first reports about plants producing adventitious root buds (shoot buds that form on roots) date back to the 19th century (Irmisch, 1850). The formation of adventitious root buds is often conditioned by plant damage and is a

differentiating trait between plants that sprout spontaneously from roots and plants that sprout only after aboveground biomass removal (Klimešová and Martínková, 2004; Bartušková et al., 2021). There are also plants that are fully dependent on adventitious root buds and root sprouting (e.g., mycoheterotrophic plants and Podostemaceae river weeds); they do not produce primary shoots, only adventitious shoots from roots (Klimešová, 2007; Macnair, 2007). Currently, a list of root sprouters is only available for one flora—the temperate flora of Europe, and based on this list, approximately 10% of the species are capable of root sprouting (RS) (Bartušková et al., 2021). While obligatory RS is rare, approximately half of the RS species sprout roots spontaneously, and the remainder sprout only after injury (Bartušková et al., 2021).

Sprouting from adventitious root buds is not only a way to survive injury, but also a vegetative mode to multiply clonally, similar to the rhizomatous or stoloniferous propagation of other clonal species via stem axillary buds (Guerrero-Campo et al., 2006; Klimešová and Herben, 2015; Van Drunen and Husband, 2019). These two types of clonal growth, i.e., adventitious root bud-based versus axillary bud-based clonality, represent two evolutionarily independent pathways (Groff and Kaplan, 1988; Klimešová et al., 2021). The presence and vigor of RS differ even among closely related species (Palacio et al., 2007; Martínková et al., 2016; Bartušková et al., 2021), and the RS ability is relatively easy to lose or gain during evolution (Klimešová et al., 2017). Despite the similarity in the appearance of plants with adventitiously sprouting from horizontal roots and those with axillary branching from rhizomes, these two clonal growth types differ in ecology. Root sprouters are less capable of sharing resources in a heterogeneous soil environment (Martínková et al., 2018), but they are better at surviving severe injury than rhizomatous herbs (Klimešová et al., 2017).

The notion that root sprouters are well adapted to disturbance, although their initial investments in bud-bearing and storage organs are relatively small (Iwasa and Kubo, 1997; Suzuki, Stuffer, 1999; Vesk and Westoby, 2004; Malíková et al., 2010; Ottaviani et al., 2020; Wu et al., 2020; Bartušková et al., 2021), raises the question of why such a valuable ability as RS is not more common among plants. An ecological reason for a low occurrence of RS ability might be a poor competitive ability of RS species due to limited translocation of resources within a clone (Martínková et al., 2018), resulting in specialization for disturbed habitats (Bond and Midgley, 2001; Del Tredici, 2001; Klimešová et al., 2017). Another possible reason is that root sprouters must overcome some developmental or physiological barriers such as the absence of root lateral meristems (Rauh, 1937; Bartušková et al., 2021) and lack of responsible phytohomonal signals (Kamada et al., 1995; Chao et al., 2006; Druege et al., 2019).

Plant development, growth, reproduction, lifespan, growth form, and vegetative regeneration—all these components of plant life and form can be modified by external environmental factors, differences in developmental competencies, and by very complex internal control of many phytohormones and phytohormonal functional groups. While many researchers have studied hormonal interactions and biochemical regulatory networks involved in developmental processes at the molecular, cellular and tissue levels (Duclercq et al., 2011; Müller and Leyser, 2011; Hill and Schaller, 2013; Motte et al., 2014; Schaller et al., 2015), the ecological consequences of phytohormonal effects under different environmental conditions and differences in phytohormonal profiles among plant strategies and forms have rarely been studied (Chao et al., 2007; Andenson et al., 2012, Doğramacı et al., 2015). Skoog and Miller (1957) found that a high auxins-to-cytokinins ratio leads to root formation, while a high cytokinin-to-auxin ratio induces shoot regeneration. Since that time, auxins and cytokinins have been considered major phytohormones for plant development. In their review, Su and Zhang (2014) noted that the polar transport of auxin and thus its asymmetric distribution is required for shoot apical meristem formation and that high levels of exogenous auxin stimulate root regeneration but inhibit shoot regeneration. In releasing adventitious buds from dormancy, phytohormones, and their interactions with environmental factors are inevitably also involved (Horvath et al., 2003; Chao et al., 2006). Therefore, endogenous auxins, cytokinins and the auxins-to-cytokinins ratio might play a critical role in RS control (Winton, 1968; Wolter, 1968; Guo et al., 2017). Regarding regeneration after injury, interactions between cytokinins and auxins have also been found to be critical (reviewed by, e.g., Duclercq et al., 2011; Motte et al., 2014; Schaller et al., 2015). Moreover, other phytohormones, such as strigolactones (Vanstraelen and Benková, 2012), abscisic acid (reviewed by Su and Zhang, 2014), and gibberellins (Jasinski et al., 2005; Chao et al., 2016), were reported to influence shoot regeneration. Therefore, phytohormones (probably mostly the auxins-to-cytokinins ratio) likely play an important role in determining RS ability and are also influenced by biomass removal. However, how RS and non-RS clonal species differ in phytohormonal profiles, contents, and ratios is unknown, and this information might shed light on the reason why some plants are capable of RS while others are not.

In this study, we experimentally exposed two closely related clonal *Inula* species (Asteraceae) differing in RS ability (root-sprouting *I. britannica* and non-root-sprouting *I. salicina*) to severe aboveground biomass removal as a potential trigger or booster of RS and asked: Do growth traits and phytohormones change when either species is injured? We hypothesized that injury will stimulate the production of adventitious root buds in RS species and that injury will lead to increased metabolism of root carbohydrate reserves, measurable as root respiration, necessary to generate new aboveground biomass. Furthermore, we expected that intact RS species would have different phytohormone profiles and contents, especially a lower auxins-to-cytokinins ratio than non-RS species, and this

difference would be even more pronounced after severe biomass removal. By testing these hypotheses, we executed the first analysis of possible drivers of root-sprouting ability in plants.

MATERIALS AND METHODS

Experimental design

We conducted a greenhouse growth experiment with two closely related herbs, clonal, spontaneously root-sprouting *Inula britannica* L. and rhizomatous, nonroot-sprouting *Inula salicina* L. (Asteraceae) (Figure 1; Clo-Pla Database, Klimešová and de Bello, 2009). While RS *I. britannica* often prefers disturbed habitats such as roadsides or water banks, non-RS *I. salicina* grows in light deciduous forests, thickets, and meadows (Pladias, 2022). These two *Inula* species were used to test how growth traits, root respiration, and phytohormonal profiles are affected by severe aboveground biomass removal. In January 2019, seeds of both species were purchased from Planta Naturalis (Markvartice u Sobotky, Czech Republic; 50.429°N, 15.199°E), which provides field-cultivated seeds, which therefore originate from many mother plants and represent several genotypes per species. Seeds were stored in a laboratory at room temperature. In February 2019, seeds were sown on wet sand in Petri dishes, then stratified in the dark at 2°C. After 4 weeks, the seeds in Petri dishes were transferred to a growth chamber (day, 23°C, 15 h; night, 16°C) to germinate. Five-day-old seedlings of both species (one seedling per pot, totally 110 pots per species) were transplanted to plastic pots (2.2 L volume) filled with a 3:2 mixture (v/v) of sand and the commercial organic substrate Zahradnický substrát (mixture of peat, humus, dolomitic limestone and sand; AGRO CS a.s., Říkov, Czech Republic) and cultivated in an unheated, naturally lit greenhouse at the Institute of Botany, Třeboň, Czech Republic (49.006°N, 14.772°E). Standard liquid NPK solution (Kristalon, 0.5-0.1-0.07 g/L of the substrate; AGRO CS a.s.) was supplied every 3 weeks, and plants were watered with tap water when necessary.

After 3 months of cultivation, at the beginning of July, half of the plants were injured; i.e., total aboveground biomass was removed at 1 cm above the topsoil level. The remaining plants were left intact as controls. The size of plants, i.e., aboveground and belowground biomass, on the day of injury ("day 0") is shown in Figure 2. Each plant from both groups was subsequently randomly assigned to one of five consequent harvests at 5-day intervals starting at day 0; i.e., control and injured plants were harvested on the 0^{th}, 5^{th}, 10^{th}, 15^{th}, and 20^{th} days after injury. Therefore, 11 replicates were harvested per harvest and treatment (control vs. injury) for each species. From these 11 replicates, biomass, belowground to aboveground biomass ratio (B:A ratio) and root respiration were determined for four plants; bud sizes were measured and number of sprouts counted for three other plants; and phytohormones were analyzed for four other plants as described later. This design allowed us to analyze a relatively large set of characteristics in time steps to compare the reaction of RS vs. non-RS species to severe aboveground biomass removal.

Biomass and bud traits

At each harvest, the belowground and aboveground biomass of four plants of both species from each treatment (control vs. injury) was dried at 80°C to a constant mass, then the B:A biomass ratio was calculated for both species. The number of adventitious root buds (shoot buds that formed

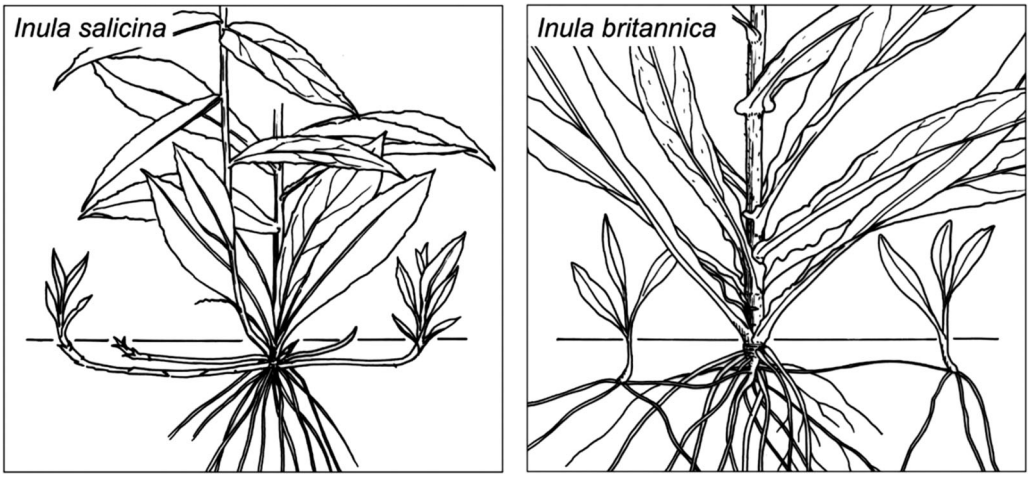

FIGURE 1 *Inula salicina* (left) is a clonal, nonroot-sprouting herb, which forms hypogeogeneous horizontal rhizomes (belowground stems), producing daughter ramets. After injury (in the present study, removing the majority of aboveground biomass), it regenerates from belowground axillary buds on rhizomes or the basal part of a stem. *Inula britannica* (right) is a clonal, spontaneously root-sprouting herb, which forms adventitious buds on roots, that grow into daughter ramets. After the removal of all stem parts, this species regenerates from adventitious root buds.

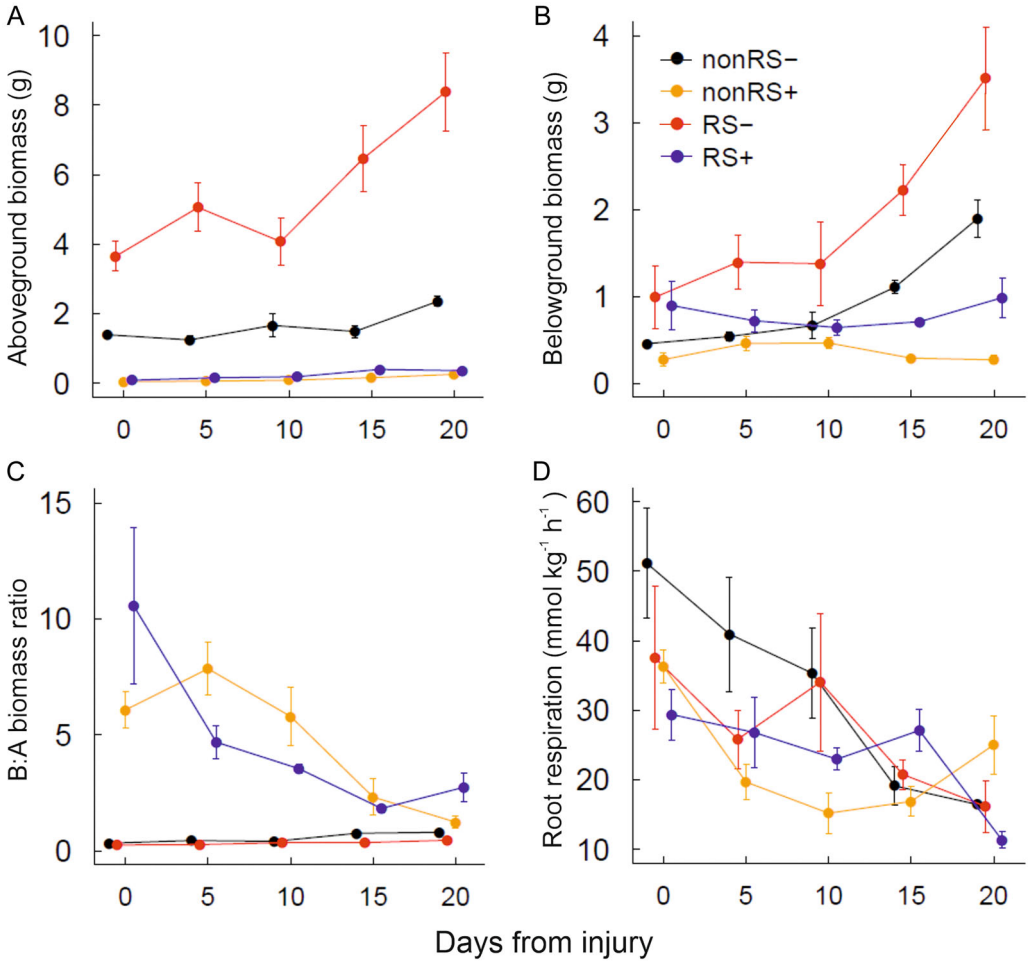

FIGURE 2 (A) Aboveground and (B) belowground biomass, (C) belowground-to-aboveground biomass ratio (B:A ratio), and (D) aerobic root respiration in plants harvested in 5-day intervals. Means and standard errors are shown for plants of nonroot-sprouting *Inula salicina* (nonRS) and root-sprouting *Inula britannica* (RS), either severely injured (+) or not (−). For statistical results, see Table 1.

on roots) and root sprouts were counted only for the RS species. All new root sprouts (new shoots elongating and growing from root buds) on the entire root system were counted. The adventitious root buds were counted and bud length and diameter measured for each of three randomly selected 5-cm-long root fragments divided into three portions (proximal, middle, and distal). Each portion was cross-sectioned once. Cross sections (20–50 μm thick) were obtained using a sliding lab microtome, clarified with sodium hypochlorite solution, stained with safranin and Astra blue (Bukatsch, 1972), and mounted on glass slides in 50% v/v aqueous glycerin. The slides were examined using an Olympus BX53 microscope, Olympus DP73 camera, and Olympus cellSens Entry 1.9 software. Quantitative measurements were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The number of buds was obtained by counting all buds at different developmental stages that were present in the cross sections. Bud length and diameter were measured for each bud, and mean values were calculated per plant. Bud diameter was the distance between cataphylls on the bud base, and bud length was the length between the cataphyll tip and the center of the stele.

Aerobic root respiration

As a criterion of root metabolic activity (Adamec, 2005), the aerobic respiration rate (root respiration) was measured for roots of four control and four injured plants of both species at 5-day intervals, starting on the day of injury (5 h after biomass removal). Root respiration was measured in 2-cm root segments excised 5 mm below the root crown. Five to nine root segments per plant (60–200 mg fresh biomass; 8–44 mg dry biomass) were used for individual measurements. Oxygen-based respiration rate of roots was measured using a Clark-type O_2 sensor and a chart recorder in a diluted solution (0.5 mM KCl + 0.1 mM $CaCl_2$, pH ca. 5.5) in an 8.8-mL stirred thermostatted chamber at 20.0 ± 0.1°C in darkness (Adamec 2005). Each measurement lasted 15–20 min, and the O_2 concentration was approximately 70–90% of the saturation during the measurements. Root

respiration was expressed as mmol kg^{-1} h^{-1} recalculated to biomass dry mass.

Phytohormone analysis

Plant hormones in roots were quantitatively determined as described previously (Prerostová et al., 2021) using four replicates per species and treatment for each analysis. Deep-frozen root samples (representative samples of the whole root system were put into liquid nitrogen immediately after substrate was washed off) were subsequently lyophilized (in aliquots of 10–20 mg) and homogenized in 2-mL micro-centrifuge tubes with 50 µL cold extraction solvent (1 M formic acid) and 10 µL isotope-labelled standards in FastPrep-24 (MP Biomedicals, Eschwege, Germany) at 6 m/s for 40 s. After centrifugation (4°C, 30,000 × *g*), the supernatants were collected and applied to SPE Oasis HLB 96-well column plates (10 mg/well; Waters, Milford, MA, USA), activated with 100 µL methanol, and then eluted with 100 µL 50% acetonitrile using s Pressure+ 96 manifold (Biotage, Uppsala, Sweden). The pellets were re-extracted in 100-µL portions of 50% acetonitrile, centrifuged, and applied again to the column plates.

Phytohormones in each eluate (injected in technical duplicates) were separated on a Kinetex EVO C18 column (2.6 µm, 150 × 2.1 mm, Phenomenex, Torrance, CA, USA). Mobile phases consisted of (A) 5 mM ammonium acetate in water and (B) 95:5 acetonitrile/water (v/v). The following gradient program was applied: 5% B in 0 min, 7% B in 0.1 to 5 min, 10 to 35% in 5.1 to 12 min, 100% B at 13 to 14 min, and 5% B at 14.1 min. Hormones were analyzed on an LC–MS system consisting of a UHPLC 1290 Infinity II (Agilent, Santa Clara, CA, USA) coupled to a 6495 Triple Quadrupole mass spectrometer (Agilent). Mass spectrometric analysis was performed in multiple reaction monitoring mode using the isotope dilution method. Data acquisition and processing were carried out with Mass Hunter software B.08 (Agilent). Phytohormones were quantified as the amount (in picomoles) per gram of root dry mass. All analyzed individual phytohormones were classified into six functional groups (auxins, cytokinins, ABA types, gibberellins, jasmonates, phenolics), and cytokinins were classified into seven categories based on conjugation status (free bases, ribosides, *N*7-glucosides, *N*9-glucosides, *O*-glucosides, phosphates and methylthio derivatives; Appendix S1).

Statistical analyses

All statistical analyses were performed in R v 3.6.0 (R Core Team, 2019) using the packages mvabund (v 4.0.1, Wang et al., 2012) and vegan (v 2.5-6, Oksanen et al., 2019). To test whether the aboveground and belowground biomass, the B:A ratio, and root respiration were affected by RS ability, injury and sampling date (time), we applied a linear model. For the sampling date, we set Helmert contrasts that are useful to examine trends in ordinal variables. We also tested the significance of interactions between RS ability, injury, and sampling date following backward selection (i.e., starting with the most complex model and then omitting all nonsignificant terms).

Two separate multivariate linear models were performed to test the effects of RS ability, injury, and sampling date on phytohormones (Wang et al., 2012). The first one was performed on six functional groups of phytohormones (cytokinins, ABA metabolites, gibberellins, jasmonates, auxins, phenolics) and seven cytokinins groups (free bases, ribosides, *N*7-glucosides, *N*9-glucosides, *O*-glucosides, phosphates, methylthio derivatives). The second linear model was performed on auxins and cytokinins content and auxins to cytokinins ratio. All values were standardized and log-transformed before the analyses. The significance tests were based on the likelihood ratio (LR) test and 999 permutations. Multivariate patterns in phytohormones were projected using redundancy analysis (RDA) ordination diagrams.

RESULTS

Biomass, buds, and root respiration

The RS species (*I. britannica*) was larger than the non-RS species (*I. salicina*) with a higher aboveground and belowground biomass (Table 1; Figure 2A, B). However, species did not differ in B:A biomass ratio and root respiration (Table 1; Figure 2C, D). Injury and sampling date affected all variables (Table 1, Figure 2). Generally, injury decreased aboveground and belowground biomass and increased the B:A ratio (Table 1, Figure 2); with sampling date, above and belowground biomass increased, while B:A ratio and root respiration decreased (Table 1, Figure 2; Appendix S2). Neither the number of sprouts and adventitious root buds nor bud diameter and length were influenced by injury and sampling date; i.e., the injury did not boost RS in RS species *I. britannica* (Appendix S3).

Phytohormones

RS ability, injury, and sampling date had a significant effect on the contents of functional groups of phytohormones and cytokinins groups (Tables 2, 3). RS vs. non-RS plants differed more than injured vs. non-injured plants in contents of both functional and cytokinins groups, i.e., RS ability had a much stronger effect on phytohormonal profiles than injury did (Tables 2–4). Regarding the phytohormonal functional groups, the RS species produced more cytokinins and gibberellins, while the non-RS species contained more ABA metabolites, jasmonates, auxins, and phenolics (Table 4, Figure 3). Injury increased the content of cytokinins, auxins, and phenolics (Table 4). For

TABLE 1 Effects of root-sprouting ability (RS ability), sampling date, injury, and their interactions on (A) aboveground biomass, (B) belowground biomass, (C) belowground-to-aboveground biomass ratio (B:A biomass), and (D) aerobic root respiration (root respiration). Significance levels: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s., nonsignificant. Only significant interactions ($P < 0.05$) are indicated. The coefficient of determination (R^2) refers to the percentage of variability explained by all tested variables.

Variable	*F*	df	R^2 (%)
A. Aboveground biomass		73	66.4
RS ability	**38.8*** **	1	
Sampling date	**3.0*** **	4	
Injury	**111.3***	1	
B. Belowground biomass			68.5
RS ability	**40.3*** **	1	
Sampling date	**10.8*** **	4	
Injury	**58.0*** **	1	
Sampling date × Injury	**10.0*** **	4	
C. B:A biomass			67.0
RS ability	n.s.	1	
Sampling date	**7.4*** **	4	
Injury	**98.1*** **	1	
Sampling date × RS ability	**2.6***	4	
Sampling date × Injury	**9.0*** **	4	
D. Root respiration			32.2
RS ability	n.s.	1	
Sampling date	**8.8*** **	4	
Injury	**7.3** **	1	

TABLE 2 Results of likelihood ratio (LR) test of effect of root-sprouting ability (RS ability), injury, and sampling date on (A) contents of phytohormonal functional groups and (B) cytokinins groups. For detailed descriptions of the groups, see Materials and Methods and Appendix S1. Significance level: **$P < 0.01$. The coefficient of determination (R^2) refers to the percentage of variability explained by all tested variables.

Variable	LR	df	R^2 (%)
A. Functional groups		79	42.0
RS ability	**126.6** **	1	
Injury	**91.2** **	1	
Sampling date	**82.2** **	4	
B. Cytokinins groups		79	43.8
RS ability	**192.3** **	1	
Injury	**103.1** **	1	
Sampling date	**63.5** **	4	

TABLE 3 Results of likelihood ratio (LR) test of effect of root-sprouting ability (RS ability), injury, and sampling date on contents of (A) individual phytohormonal functional groups and (B) cytokinins groups. For detailed descriptions of groups, see Materials and Methods and Appendix S1. Significance levels: **$P < 0.01$; *$P < 0.05$; n.s., nonsignificant.

Variable	Total df = 79	RS ability df = 1	Injury df = 1	Sampling date df = 4
A. Functional groups				
	Cytokinins	**56.7** **	**31.0** **	**16.3** **
	ABA metabolites	**6.7***	n.s.	**22.9** **
	Gibberellins	**9.9** **	n.s.	**10.5** **
	Jasmonates	**13.7** **	n.s.	**20.3** **
	Auxins	**5.0***	**7.9***	n.s.
	Phenolics	**34.6** **	**44.4** **	**9.2** **
B. Cytokinin groups				
	Free bases	**4.9***	n.s.	**13.6** **
	Ribosides	**28.0** **	n.s.	**16.0** **
	***N*7-glucosides**	**6.1***	**17.9** **	**7.7***
	***N*9-glucosides**	**43.4** **	**18.3** **	**5.7***
	***O*-glucosides**	**74.6** **	**27.1** **	**8.7***
	Phosphates	**4.4***	n.s.	**11.4** **
	Methylthio derivatives	**31.0** **	**34.3** **	n.s.

cytokinins, the RS species produced more ribosides, *N*9-glucosides, *O*-glucosides, and phosphates, while the non-RS species produced more free bases, *N*7-glucosides, and methylthio derivatives (Table 4, Figure 4). Injury increased the production of all glucosides and methylthio derivatives (Table 4).

The ratio of auxins and cytokinins

The ratio of auxins and cytokinins (aux/cyt ratio) was significantly influenced by RS ability and by the interaction of RS ability and injury (Table 5, Figure 5A), whereas it was not affected by sampling date (Table 5). The aux/cyt ratio was significantly lower in the RS species, and injury further decreased it. In contrast, in the non-RS species, injury had an opposite effect on the ratio, and the ratio increased afterward (Figure 5A). Differences in the contents of both auxins and cytokinins lay behind the differences in the aux/cyt ratio (Table 5; Figure 5B, C). While the injury similarly increased cytokinins in both species, auxins were increased by the injury only in the non-RS species (Figure 5B, C). Intact RS individuals did not differ from injured individuals in auxin contents, but cytokinins were higher in injured than in intact RS plants (Figure 5B, C).

TABLE 4 Effect of root-sprouting ability and injury on contents of (A) individual phytohormonal functional groups and (B) cytokinin groups based on analyses in Table 3. Root-sprouting (RS) vs. nonroot-sprouting (non-RS) individuals and injured vs. intact individuals were compared. For detailed descriptions of groups, see Methods and Appendix S1. n.s., no significant difference between treatments; +, content significantly higher than in the counterpart individual.

Variable	RS	non-RS	Injured	Intact
A. Functional groups				
Cytokinins	+		+	
ABA metabolites		+	n.s.	n.s.
Gibberellins	+		n.s.	n.s.
Jasmonates		+	n.s.	n.s.
Auxins		+	+	
Phenolics		+	+	
B. Cytokinin groups				
Free bases		+	n.s.	n.s.
Ribosides	+		n.s.	n.s.
***N*7-glucosides**		+	+	
***N*9-glucosides**	+		+	
***O*-glucosides**	+		+	
Phosphates	+		n.s.	n.s.
Methylthio derivatives		+	+	

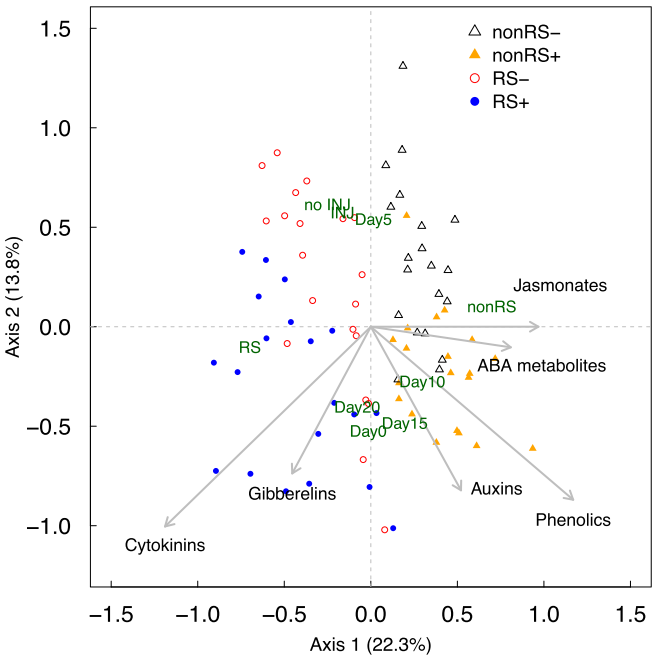

FIGURE 3 RDA ordination diagram showing relationships among phytohormonal functional groups (phytohormone contents, grey arrows), sampling time (day 0–20), and injury (INJ: injury applied, no INJ: control, intact plants) for nonroot-sprouting herb *Inula salicina* (nonRS) and root-sprouting herb *Inula britannica* (RS), either severely injured (+) or not (−). The first two ordination axes are shown, together explaining 36.1% of the variability in the data set.

DISCUSSION

In this study, by comparing growth variables and phytohormonal profiles of two closely related clonal herbs differing in their root-sprouting ability (root-sprouting *I. britannica* and rhizomatous *I. salicina*), we analyzed possible drivers or regulatory factors of root-sprouting ability. We confirmed RS ability in the previously reported RS species, *I. britannica*; however, contrary to our expectations, RS ability was not boosted by injury. While root respiration did not differ between species and decreased continuously with time in the majority of treatments, phytohormone profiles differed significantly. In this study, we supported the common but never tested view that RS is enabled by a low auxins-to-cytokinins ratio. Our results suggest that intrinsic phytohormone regulation might be behind the ability of RS and that injury (causing phytohormonal imbalance) is less important in the spontaneously RS species, *I. britannica*. Our study proposed, albeit on limited material, possible drivers behind the existence of different growth forms in plants.

RS has been reported to be advantageous in severely disturbed habitats because it increases the fitness and probability that an individual and population will persist after frequent and severe biomass removal or in habitats lacking seed banks (Martínková and Klimešová, 2016; Martínková et al., 2016, 2021). However, in our study, we found that injury affects

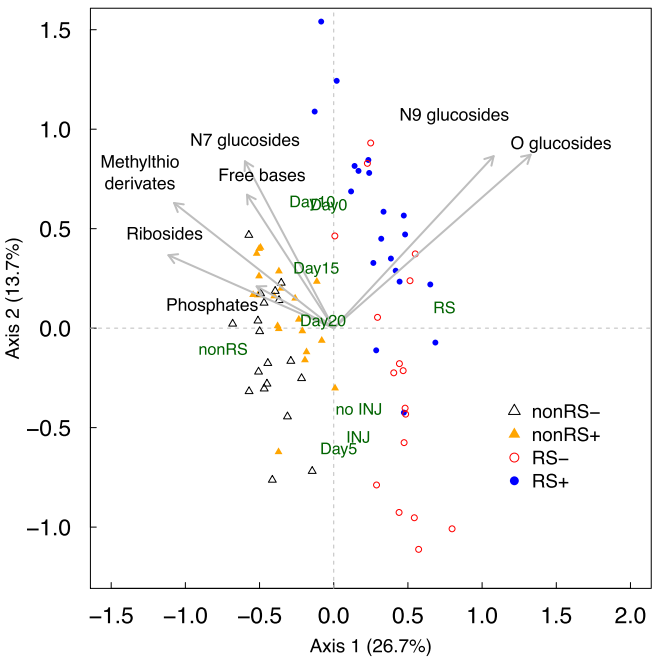

FIGURE 4 RDA ordination diagram showing relationships among cytokinin groups (phytohormone contents, grey arrows), sampling time (day 0–20), and injury (INJ: injury applied, no INJ: control, intact plants) for the non-root-sprouting herb *Inula salicina* (nonRS) and root-sprouting herb *Inula britannica* (RS), with or without severe injury (+) (−). The first two ordination axes are shown, together explaining 40.4% of the variability in the data set

TABLE 5 Effect of root-sprouting ability (RS ability), injury, and their interactions on the auxins-to-cytokinins ratio (aux/cyt ratio) and contents of auxins and cytokinins. The results of *F* test, degrees of freedom, and significance are shown. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s., nonsignificant. The coefficient of determination (R^2) refers to the percentage of explained variability by all tested variables.

Variable	*F*	df	R^2 (%)
A. Aux/cyt ratio		76	60.0
RS ability	**94.2*** **	1	
Injury	n.s.	1	
Sampling date	n.s.	1	
RS ability × Injury	**27.0*** **	1	
B. Auxins		76	22.9
RS ability	**6.2***	1	
Injury	**9.1****	1	
Sampling date	**n.s.**	1	
RS ability × Injury	**11.2****	1	
C. Cytokinins		76	68.1
RS ability	**126.0*** **	1	
Injury	**39.3*** **	1	
Sampling date	**n.s.**	1	
RS ability × Injury	**6.8***	1	

neither the number of adventitious root buds nor the number of new sprouts in the RS species. The reason might be that in *I. britannica*, RS occurs as a standard part of its life cycle, allowing vegetative multiplication and horizontal occupation of space similar to clonal rhizomatous herbs. Clonal herbs with clonality based on roots (root sprouters) occur more frequently in severely disturbed habitats than clonal herbs with stem-based clonality (e.g., rhizomatous herbs, stoloniferous) (Klimešová et al., 2017). Even though both stem- and root-derived clonality have buds belowground and thus the plants are potentially similarly successful at regeneration after severe aboveground biomass removal, the proportion of clonal root sprouters is higher when disturbance pressure is higher (Klimešová et al., 2017).

In our experiment, 4-month-old RS individuals had already formed several root shoots; i.e., several potentially independent ramets, while the non-RS had not formed any rhizomes yet. This developmental advantage might account for the higher proportion of clonal RS herbs in more disturbed habitats. Disturbed habitats are frequently characterized by the low predictability of disturbance (Bellingham and Sparrow, 2000; Schippers et al., 2001; Seifan et al., 2013). The readiness of RS species to form ramets already in early ontogeny might be advantageous after a disturbance at very early life stages. The second

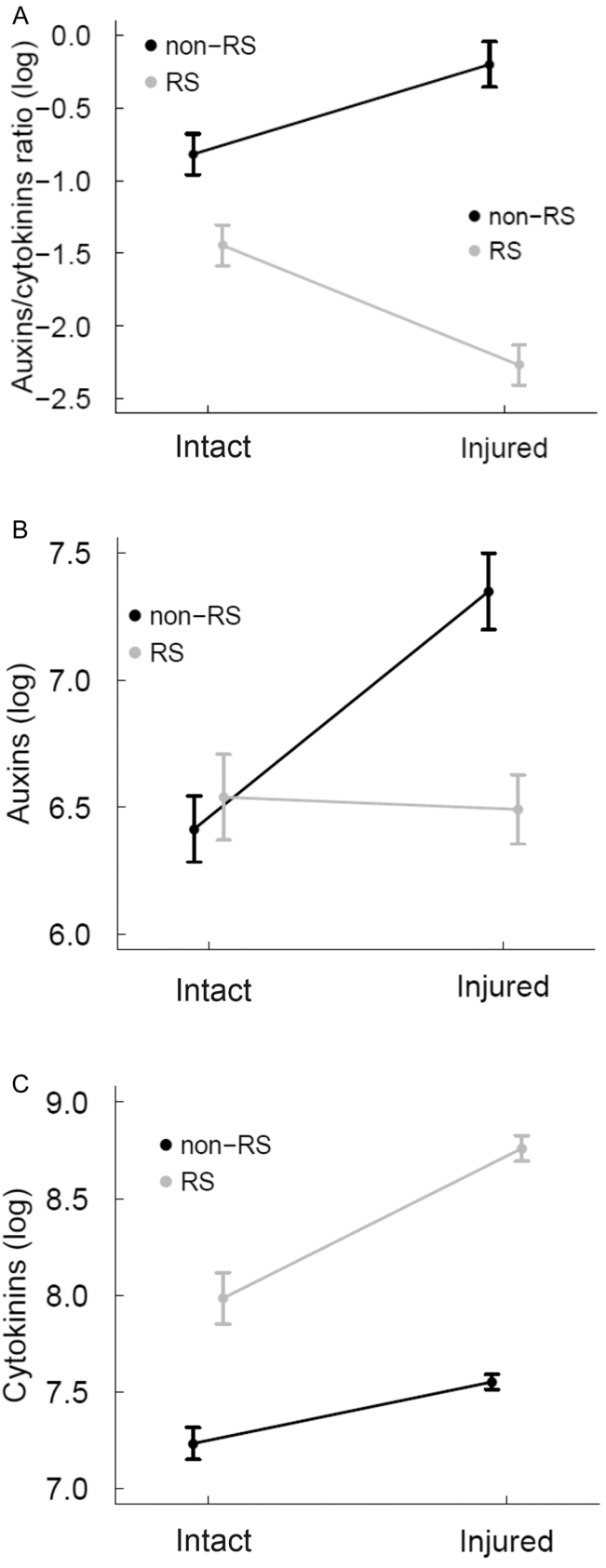

FIGURE 5 (See caption on next page)

explanation might be that severe disturbance frequently causes belowground system fragmentation (Pausas et al., 2018; Ott et al., 2019). When rhizomes are fragmented, at least one whole module with an axillary bud is necessary for successful regeneration. In RS species, there is no such morphological limitation because adventitious buds can be formed anywhere on the root system and at any distance from other buds, so the length of the fragment is not as important as it is in rhizomatous species (Benot et al., 2010; Cornelissen et al., 2014; Herben and Klimešová, 2020).

The relatively higher investment in root biomass by RS species in later stages of the experiment, irrespective of injury, might be due to the role of roots as storage organs in RS species. Carbohydrate reserves stored in plant organs are important for the growth and maintenance of plant individuals (Salomón et al., 2015). Storage organs such as rhizomes, coarse roots, bulbs, or tubers are commonly belowground because there they are safe from the majority of disturbance types. After total aboveground destruction, the rebuilding of green biomass is entirely dependent on stored reserves in these belowground organs (Wyka, 1999; Drake et al., 2009; Zhu et al., 2014). In our study, both tested species had only roots belowground at the time of biomass removal; no rhizomes were formed in *I. salicina* yet. The initial regrowth of injured plants thus required the mobilization of the carbohydrates stored in roots in both species. We expected intensified respiration, especially in the RS species because increased root respiration rate can serve as a marker for the intensified usage of root reserves (Volenec et al., 1996; Aubrey et al., 2012; Salomón et al., 2015). However, we found no signals of intensified root respiration, neither after several hours from injury (Figure 2, day 0) nor a difference in root respiration between species. The reason for the lack of increased respiration in injured individuals might lie in an insufficient amount of reserves available for more intensive respiration due to young plant age. Such young plants, even though they have the habit of adult ones, probably invest a majority of photosynthates into intensive organ growth rather than storage. This view is supported by the lack of such reserves in the roots of *I. britannica* and *I. salicina* at the same age (4 months; Filartiga et al., 2022).

Both species significantly differed in phytohormonal profiles in our study. Importantly, the RS species had a lower auxins-to-cytokinins ratio than in the non-RS species, and injury decreased the ratio in the RS species even more. The difference in the ratio is due to the higher cytokinins in the RS species and lack of increase in auxins in the RS species after the injury. Therefore, the interplay of auxins and cytokinins, i.e., a low auxins-to-cytokinins ratio, plays a key role in RS occurrence. Moreover, injury causing a further decrease in the ratio, might lie behind the triggering or boosting effect of an injury found in other RS species (Guo et al., 2017). Also, gibberellins might be an important contributor to RS ability because they were significantly higher in RS species than in non-RS species. Gibberellins are responsible for the budding and breakdown of starch to glucose in germinating seeds (Swain and Singh, 2005; Sun et al., 2019) and belowground storage organs as a source of energy for growth (Rentzsch et al., 2012; Sonnewald and Sonnewald, 2014). However, the phytohormonal control of RS ability is even more complex. Some of the cytokinin types were higher in the RS species (ribosides, *N*9 glucosides, *O* glucosides, and phosphates), and others higher in the non-RS species (*N*7 glucosides and methylthio derivatives). Our results also suggest that the role of other hormones might be less important in controlling RS ability, even though the stress hormones jasmonates and ABA metabolites are synthesized under various stresses such as drought or wounding (Suttle et al., 2013; Savchenko et al., 2014; Bruňáková et al., 2015). But this possibility needs further exploration.

FIGURE 5 (A) Auxins-to-cytokinins ratio and (B) contents of auxins and cytokinins (C) in pmol/g of dry mass at the end of the experiment (on day 20 after injury) are displayed for the nonroot-sprouting herb *Inula salicina* (nonRS) and root-sprouting herb *Inula britannica* (RS), either severely injured or not. Means and standard errors of log-transformed values are shown. Statistical significance of the effect of root-sprouting ability (RS ability), injury, and their interactions on the auxins-to-cytokinins ratio and contents of auxins and cytokinins: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; n.s., not significant. For detailed statistical results, see Table 5.

CONCLUSIONS

This first study of possible drivers of different plant growth forms of a root-sprouting clonal herb and a non-RS rhizomatous herb showed that a low auxins-to-cytokinins ratio might contribute to the RS ability and that gibberellins might also play an important role. Injury was less important in the spontaneously RS species, *Inula britanica*. Even though these results were obtained for only two species that differed in RS ability, they provide valuable pioneering insight into the control of RS ability and a baseline for future studies. The future direction of studies on RS ability might include (1) the application of methods used in this study to more than one related pair of RS vs. non-RS species to corroborate our findings, (2) comparison of pairs of spontaneously RS species vs. species root-spouting only after injury to test the effect of injury in these two distinct RS strategies, and (3) identification of genes driving RS ability. Altogether, these three approaches would provide a more complex and comprehensive assessment of RS ability, an alternative strategy to stem-derived clonality, tentatively more advantageous in conditions of severe disturbances.

AUTHOR CONTRIBUTIONS

J.M. designed and conducted the experiment, co-formulated the idea, performed biomass analysis, and wrote the manuscript; J.K. formulated the original idea and co-wrote the manuscript; V.M., P.I.D., R.F., A.G., and J.L. performed phytohormone analyses; M.B. analyzed data; L.A. performed the analysis of root respiration, A.F. performed the analysis of adventitious root buds.

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DATA AVAILABILITY STATEMENT

All data and code associated with this paper are publicly available on Mendeley Data at https://doi.org/10.17632/jwdwvyyj2w.2 (Jevon, 2021).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX S1. Detailed descriptions of phytohormonal groups.

APPENDIX S2. Statistical tests of trends in aboveground and belowground biomass, B:A biomass ratio, and root respiration over time.

APPENDIX S3. The number of sprouts and adventitious root buds and bud diameter and length of RS species *I. britannica*.

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