RESPONSES OF THREE *Prunus spinosa* L. ECOTYPES TO LOW TEMPERATURE UNDER CONTROLLED CONDITIONS

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Abstract

Non-native ecotypes could be at risk of low temperature damage when transplanted outside their adaptive range due to advanced sprouting status than that of the local ecotypes. Moreover, hybridization with local ecotypes might lead to a compromised (maladapted) future generation. In this study, we explored the influence of local adaptation to low temperature (frost) events at continental (German against Italian) and regional (two German ecotypes: Brandenburg (BB) and Rheinland-Pfalz (RPF) scales. Plants were sourced as cuttings and co-cultivated on the container area of Leibniz University of Hannover, Germany, for at least two years. In April 2013 and 2014 randomly selected plants were treated with temperatures of -12°C and -6°C and 5°C under controlled conditions respectively. Plants treated at 5°C served as control. Plant frost injury was measured as relative electrolyte leakage (REL). Biomarkers (glucose, fructose, sucrose, starch and proline) after frost event were quantified. Regeneration after the frost events were evaluated and quantified by the number of shoots and length of the longest shoot. All the data were subjected to multiple analysis of variance using R statistical tool and means at $p \leq 0.05$ were separated by Tukey. Frost events (-12°C and -6°C) led to shoots injury (high REL values), compromised apical dominance and reduced plant height compared to control. German ecotypes did not differ significantly in damage but differed inconsistently in a few biomarkers. This implies that German ecotypes could be substituted for another in a planting program. In 2014 experiment, the Germany ecotype BB differed significantly with the Italian ecotype in growth, REL and a few biomarkers. The Italian ecotypes sprouted early than the German ecotypes thus the high REL related to phenological stage of the leaves. Nevertheless, mature leaves had lower REL than emerging or expanding leaves. The regeneration performance after frost exposure indicated that Italian ecotypes formed more shoots and were taller than the German ecotype. Imperatively the Italian ecotype could be utilised cautiously in Germany.

Keywords: Ecotypes, electrolyte leakage, freezing, phenology, regeneration

Introduction

Global warming events are hypothetically likely to increase the incidences of erratic and sporadic frost events and hence frost damage (Kim et al. 2014; Menzel et al. 2015). Frost damage occurs when, plants are exposed to low temperatures that induces ice formation inside the plants' tissues and rapture the cells membrane. Damaged cell membranes could be quantified by relative electrolyte leakage (REL). Since the integrity of the cell is compromised, the cell contents diffuse into an ion free solution and could be measured as a change in electrical conductivity (Verslues et al. 2006).

When spring frost is considered, plants that sprout early are reported to have a high likelihood to suffer damage than those that sprout later (Augspurger 2009; Myking and Heide 1995). This is because of their tender leaves coinciding with low temperature during spring. Conversely, when temperature are warming up (above 5 °C), leaves of such plants (that sprout earlier than others) could mature early and are likely to sustain damage than those that sprout later (Kreyling et al. 2012) when freezing temperature occur late in spring. Moreover, they are likely to recover faster after frost damage (Menzel et al. 2015). Furthermore, It has been postulated that plants that sprout early could have formed lignin in their leaves cell wall hence better able to resist freezing temperatures (Boudet et al. 1995). It has also been argued that early sprouting could have photosynthetic advantages and hence early growth (Menzel and Fabian 1999; Menzel et al. 2015). Some of the photosynthetic sugars could contribute immensely in cryoprotection (Morin et al. 2007).

Ecologically, local ecotypes are reported to be better adapted to local climatic conditions and could quickly adjust to local conditions for their survival and performance than non-local ecotypes (De Frenne et al. 2011). Nevertheless, some non-local ecotypes are likely to adjust to new environment through diverse phenological, physiological and biochemical mechanism (Schreiber et al. 2013; Wanjiku and Bohne 2016).·These mechanisms may involve synthesis of various biomarkers (glucose, fructose, sucrose and proline) that serves as cryoprotectant in low temperatures (Morin et al. 2007; Wanjiku and Bohne 2016).

Prunus spinosa L. (commonly known as blackthorn or sloe) is an environmentally important shrub that provides food and shelter to wild animals. It is also utilized for landscaping and for extraction of various antioxidants (Ruiz-Rodríguez et al. 2014). Blackthorn has a tendency of growing along forest edges and hedges in open landscapes. It is pollinated majorly by insects. The species is widely distributed in Europe, Northern Africa and part of Asia and it is reported to have low or no genetic differentiation (Leinemann et al. 2014). It proliferates through seed, root sucker and stem cuttings (Eimert et al. 2012). Due to its popularity and resilience, is propagated and widely shipped by commercial nurseries for land reclamation and restoration. It is collected, propagated and commercially supplied by nurseries irrespective of origin. However, there are demand for local seedlings due to the perception that local ecotypes might be adapted to withstand climate changes. In this study, therefore, our major objective was to evaluate adaptability of three European *Prunus spinosa* ecotypes both at local and regional level when exposed to different freezing environmental stress under controlled conditions. They include two German ecotypes and an Italian ecotype.

In a previous study, for characterising seasonal changes for different German sloe ecotypes sloe did not vary in phenology with either latitude or altitude as the range could be small (Wanjiku and Bohne 2021). On the contrary, the two German ecotypes tended to differ in some biochemical composition in spring. At regional level, the Italian ecotype significantly differed with German ecotypes in terms of phenology, growth rate and some biochemical parameters in spring (Wanjiku and Bohne 2021). Therefore, it would be important to evaluate these ecotypes further against frost to find out how they would differ accordingly. Due to experimental logistics, frost experiment was conducted in two separate years. The local comparison evaluated in 2013, while from the regional comparison were evaluated in 2014.

Material and methods

Prunus spinosa cuttings were collected by (Leinemann et al. 2014) assisted by local forest research centres which helped in identifying native ecotypes along local forest edges of ostensibly autochthonous ecotypes in Germany and Italy (ITA). The study material used for 2013 late frost experiment were from two

German ecotypes (Fig. 1) namely Brandenburg (BB) and Rheinland-Pfalz (RPF). The German federal states differ in soil, climate and topography. Rheinland-Pfalz (RPF) is highly heterogeneous, in terms of topography and climate varying in few kilometres. Italian also differs from either of the German state in edaphic and climatic data. Hence specific climatic data from a single nearby station is not feasible representative for any ecotype and instead a range is provided (Table 1). To have a comparable database, climatic data range for BB and ITA was also used. The plants were raised from cuttings obtained and potted in 2009 and some were raised in 2011. In 2014, plant material used for this frost experiment was a German ecotype, Brandenburg (BB), and an Italian (ITA) ecotype) raised from cuttings obtained and potted in 2011. All plants were raised at Leibniz University, Hannover (52°23’34”N, 9°42’13”E, 53 m a.s.l.) and cultivated under the same environment and irrigation regimes.

Frost experiments

In April 2013 and in April 2014, late frost experiments were conducted after plants were randomly allocated to either two freezing treatments (-12°C and -6°C) or a non-freezing (control) treated at 5°C. Three shoots per plant were cut (≈ 25 cm long, including buds and emerging leaves) and immediately placed in a plastic bag. Flower petals were removed whenever present. These plant parts were then placed in various chambers according to the treatment allocated (-12°C or -6 °C) for eight hours after which they were thawed to room temperature. Temperature reduction (freezing) and later increase (thawing) to 5°C was done at the rate of 5°C h^{-1}. After treatment, 3 cm shoot’s tip was severed and used for REL determination while the remaining part was shredded and microwaved for two minutes to seizure enzymatic reaction and then further oven dried at 70°C for 72 hrs.

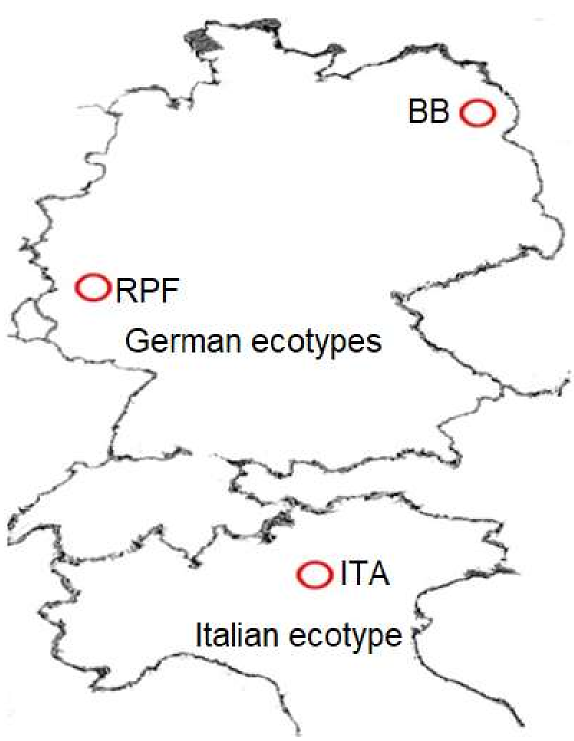

Fig. 1. Geographic locations of the investigated ecotypes (circled) of *Prunus spinosa* from Germany: Brandenburg (BB) and Rheinland-Pfalz (RPF) and Italy (ITA)

Table 1: Ecotypes' map coordinates with some ecological data. Air temperatures and rainfall data are 30 years' averages (1961 - 1990) from KlimaatlasBundesrepublik Deutschland: Karte 1.12 to 1.15 (temperature); Karte 2.12 to 2.15 (rainfall). http://www.dwd.de/bvbw/appmanager/bvbw/dwdwwwDesktop?_nfpb=true&_windowLabel=T38600134241169726338086&_urlType=action&_pageLabel=_dwdwww_klima_umwelt_ueberwachung_deutschland. Air temperatures and rainfall data [Italian (ITA)] are 12 years' average (2000 - 2012).

Origin	Altitude (m a.s.l)	Latitude	Longitude	Precipitation (mm)				Air Temp. (°C)			
				Spring	Summer	Fall	Annual	Spring	Summer	Fall	Annual
BB	44	52°38'07.2"	12°58'08.3"	120 - 140	160 - 180	100 - 120	475 - 550	8 - 9	17 - 18	9 - 10	8.5 - 9
RPF	464	50°17'22.5"	7°00'15.8"	120 - 240	180 - 240	100 - 240	700 - 1000	5 - 9	14 - 17	7 - 9	7 - 9
ITA	330 - 920	45° 43′	10° 52′	120 - 237	268 - 278	150 - 280	607 - 1008	7 - 19	16 - 29	8 - 18	7 - 18

Relative electrolyte leakage (REL)

Three shoot tips (3 cm each per plant) were severed from mother plant, rinsed with double distilled water (DDW) and immersed in 30 ml DDW vials. Infusion at room temperature was allowed to occur for 24 h. The content was then thoroughly shaken and first electric conductivity (EC) was measured. The vials were then oven heated at 70°C for 24 h and a second EC was taken. Relative electrolyte leakage (REL) was calculated as follows:

$$\text{REL} = \frac{\text{First EC}}{\text{Second EC}} \times 100$$

Carbohydrates determination

Three shoots samples (without the 3cm used for REL) were shredded, microwaved and dried at 70°C they were milled to fine powder. Ca. 30 mg of ground material, which was used to extract glucose, fructose and sucrose (GFS) determinations ensuing Zhao et al. (2010) protocols with minor modifications i.e (triethanolamine buffer (14g triethaloamine + 0.25 g $MgSO_4$ dissolved in 100 ml water, pH 7.6) and NaOH were used instead of TRIS buffer and KOH respectively). For Starch determination, the remnant pellet after GFS extraction was treated with 0.5M NAOH and heated for 30 min at 60°C. A sample of 10µl was then hydrolyzed to glucose using amyl 1, 6 glucosidase enzymes then quantified using glucose assay.

Proline determination

As a modification from Bates et al. (1973), 60 mg of milled material, was homogenized with 1.8 ml sulfosalicylic acid (3%) and incubated on ice for 30 min. The homogenates were vortexed and centrifuged at 14462xg for 15 min. Precisely 150 µl of the supernatant was treated with 90 µl acetic acid and 90 µl acid-ninhydrin (6.25 g ninhydrin powder in 60% acetic acid + 85% orthophosphoric acid at a volume ratio of 83.8 to 16.2), then boiled for 45 min. After cooling, 1.5 ml toluene (99.9 %) was added then vortexed. A sample of 0.2 ml coloured phase absorbance was determined at 520 nm using Versamax® Tuneable – Microplate reader photometer.

Regeneration plants

To assess survival and growth ability after freezing treatment, additional plants were treated in -12°C, -6 °C and 5°C (control) at the same time as the frost treatment described above. After treatments these plants were allowed to regenerate in their container outside (container area). In 2013, only BB had regeneration plants since RPF plants were used up in the previous year frost experiment. In 2014, both BB and ITA had regeneration plant each having five plants per treatment.

Statistical analysis
All data collected per ecotype and treatment was subjected to multivariate analysis of variance (MANOVA) using R statistical tool to test for treatment and ecotype main effects or interactions between and among them. To meet the requirement of a normal distribution prior to analyses, a logarithmic transformation of the data (REL, proline, glucose, fructose, sucrose and starch) was performed. Where there were no interactions, treatments and ecotype means at p ≤ 0.05 were separated by Tukey test (Pipper et al. 2012). All statistical analyses were performed using R 3.1.3 version (Rdevelopment, 2014).

Results
When the plants were sampled for late frost experiment in 2013, the two German ecotypes were at a similar sprouting stage as shown on the figure below (Fig. 2). In spring 2014, the German and Italian ecotype were at completely different stages since the Italian ecotype plants sprouted two weeks before the German (BB) ecotype (Fig. 3).

Relative electrolyte leakage
The electrolyte leakage increased with decreasing treatment temperature in 2013. H in 2014 the two freezing treatment temperature caused similar damage (Fig. 4). The two evaluated German ecotypes (BB and RPF) did not differ significantly in 2013 after treatment. Contrary, the Italian ecotype (ITA) suffered had higher leakage than German ecotype BB in the following year (2014) whether treated or not (Fig. 4). When comparing the BB ecotype in both years, the BB ecotype in had lower level of REL despite having advanced bud sprout compared to that of 2014

Fig. 2. Shoot tips of two German ecotypes (BB and RPF) of *Prunus spinosa* in April 2013 before the late frost experiment

Fig. 3. Shoots of a German (BB) and an Italian (ITA) ecotype of *Prunus spinosa* in April 2014 before the late frost experiment

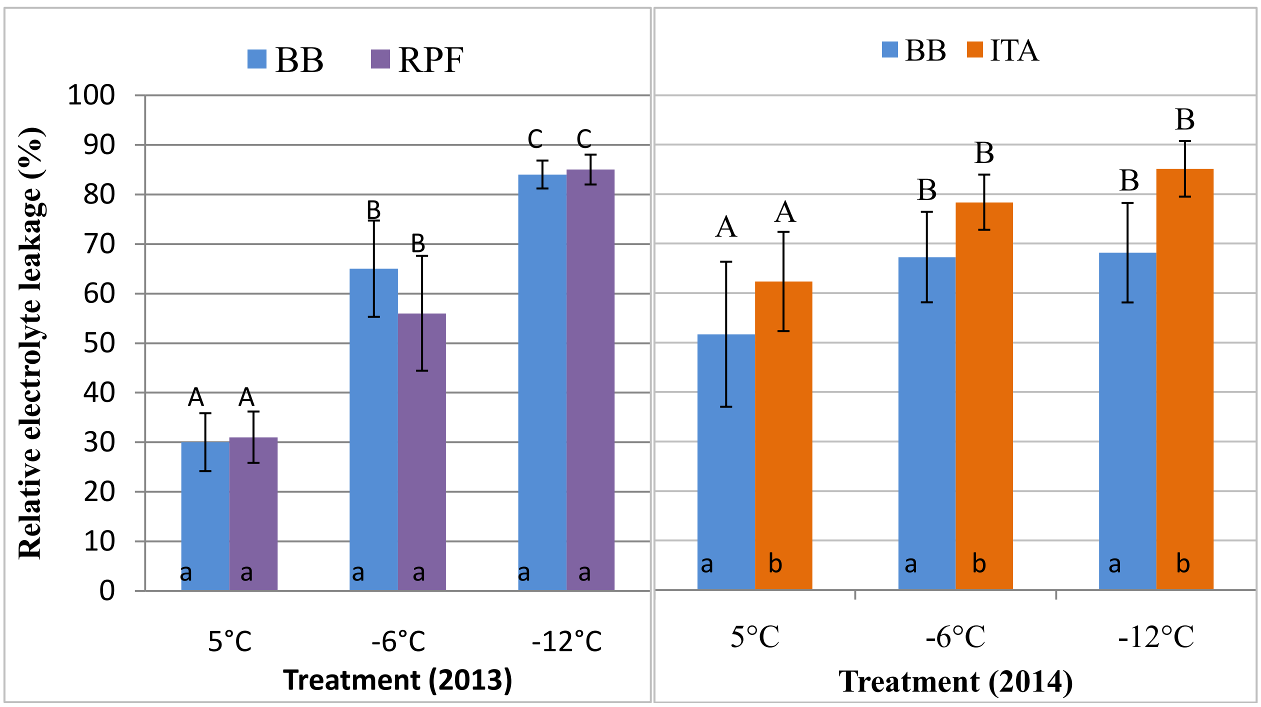

Fig. 4. Relative electrolyte leakage (%) from shoots of two German (BB= Brandenburg and RPF=RheinlandPfalz) ecotypes in April 2013 and from shoots of a German (BB) and an Italian (ITA) ecotype in April 2014 of *Prunus spinosa* in late frost experiments. Different letters show significant differences: small letters between ecotypes within a treatment, capital letters among the treatments of each ecotype. Mean ± SD, n = 8 (BB and RPF) in 2013, n = 9 (BB and ITA) in 2014

Biomarkers

When unstressed the German ecotypes BB and RPF did not differ in any of their biomarkers (glucose, fructose, sucrose, starch and proline) concentration (Table 2). However, when they were treated (-6 °C and -12°C), the RPF ecotype increased its glucose, fructose and sucrose concentration while the BB ecotype did not react in any of the sugars except a tendency to decline its sucrose concentration. Starch in this experiment did not react to any late frost treatment (Table 2). The two German ecotypes differed significantly in glucose and sucrose concentration where RPF had higher concentration than BB (Table 2).

Proline concentration increased marginally but not significant at -6°C compared to the control in both German ecotypes BB and RPF. At -12°C, the ecotype BB significantly increased its proline concentration compared to that of the control while RPF declined its proline concentration (Table 2). Due to this opposing trend at -12°C the ecotypes differed significantly with BB having up to 2.5 times higher proline concentration than that of RPF.

Comparing the German (BB) and the Italian (ITA) ecotype, in the following year (2014): when the ecotype were unstressed, they differed only in starch concentration where BB had higher concentration than ITA (Table 3). Subsequently after -6 °C treatment, there are no reactions in any of the tested biomarkers in both ecotypes. Still, the difference in starch between the ecotypes was maintained. Upon treating the ecotypes at -12°C, both ecotypes (BB and ITA) significantly increased their glucose and fructose concentration. Additionally, the Italian ecotype increased its sucrose and starch concentration (Table 3). Ecotypes differed no more in starch concentration but rather in sucrose and fructose concentration. In both incidences ITA had the highest concentration of fructose and sucrose.

Table 2: Glucose, fructose, sucrose, starch (% dry mass) and proline (µg g^{-1}) in shoots of *Prunus spinosa* ecotypes in April 2013.

Biomarker	Treatment	Ecotype	
		BB	RPF
Glucose	5 °C	0.44 ± 0.13 ^{Aa}	0.39 ± 0.10 ^{Aa}
	-6 °C	0.40 ± 0.08 ^{Aa}	0.60 ± 0.16 ^{Bb}
	-12°C	0.48 ± 0.07 ^{Aa}	0.66 ± 0.12 ^{Bb}
Fructose	5 °C	0.41 ± 0.13 ^{Aa}	0.39 ± 0.21 ^{Aa}
	-6 °C	0.38 ± 0.11 ^{Aa}	0.47 ± 0.12 ^{ABa}
	-12°C	0.39 ± 0.20 ^{Aa}	0.60 ± 0.09 ^{Ba}
Sucrose	5 °C	0.82 ± 0.31 ^{Ba}	0.90 ± 0.41 ^{Aa}
	-6 °C	0.72 ± 0.23 ^{AB}a	1.25 ± 0.30 ^{Ab}
	-12°C	0.56 ± 0.35 ^{Aa}	0.92 ± 0.25 ^{Ab}
Starch	5 °C	0.12 ± 0.11 ^{Aa}	0.22 ± 0.31 ^{Aa}
	-6 °C	0.22 ± 0.19 ^{Aa}	0.38 ± 0.30 ^{Aa}
	-12°C	0.36 ± 0.43 ^{Aa}	0.34 ± 0.34 ^{Aa}
Proline	5 °C	371 ± 143 ^{Aa}	331 ± 90 ^{ABa}
	-6 °C	442 ± 334 ^{Aa}	572 ± 263 ^{Ba}
	-12°C	659 ± 177 ^{Ba}	267 ± 143 ^{Ab}

Note: Different superscript letters show significant differences: small letters between ecotypes within a treatment, capital letters among the treatments of each ecotype. Mean ± SD, n = 8 in 2013.

Table 3: Glucose, fructose, sucrose, starch (% dry mass) and proline (µg g^{-1}) in shoots of German (BB) and Italian (ITA) ecotype of *Prunus spinosa* in April 2014.

		Ecotype	
Biomarker	Treatment	BB	ITA
Glucose	5°C	0.14 ± 0.10 $^{A\ a}$	0.16 ± 0.10 $^{A\ a}$
	-6°C	0.14 ± 0.06 $^{A\ a}$	0.19 ± 0.09 $^{AB\ a}$
	-12°C	0.26 ± 0.07 $^{B\ a}$	0.33 ± 0.21 $^{B\ a}$
Fructose	5°C	0.11 ± 0.04 $^{A\ a}$	0.1 ± 0.07 $^{A\ a}$
	-6°C	0.14 ± 0.05 $^{AB\ a}$	0.11 ± 0.06 $^{A\ a}$
	-12°C	0.19 ± 0.05 $^{B\ a}$	1.22 ± 0.79 $^{B\ b}$
Sucrose	5°C	0.2 ± 0.10 $^{A\ a}$	0.21 ± 0.11 $^{A\ a}$
	-6°C	0.22 ± 0.09 $^{A\ a}$	0.18 ± 0.09 $^{A\ a}$
	-12°C	0.21 ± 0.09 A a	0.91 ± 0.57 B b
Starch	5°C	0.73 ± 0.05 ^{Ab}	0.64 ± 0.05 ^{Aa}
	-6°C	0.72 ± 0.08 ^{Ab}	0.65 ± 0.06 ^{Aa}
	-12°C	0.74 ± 0.14 ^{Aa}	0.93 ± 0.3 ^{Ba}
Proline	5°C	312 ± 155 ^{Aa}	260 ± 85 ^{Aa}
	-6°C	352 ± 68 ^{Ab}	283 ± 65 ^{Aa}
	-12°C	369 ± 124 ^{Aa}	335 ± 74 ^{Aa}

Note: Different superscript letters show significant differences: small letters between ecotypes within a treatment, capital letters among the treatments of each ecotype. Mean ± SD, n = 9.

Proline had the tendency to marginally increase with declining temperature treatments. Nonetheless, the German ecotype significantly differed with the Italian ecotype at -6 C treatment (Table 2).

Comparing the ecotype BB in both years, in 2013 the ecotype had higher GFS and proline -12°C and lower starch compared to 2014 (Table 2 and Table 3). The REL was higher in 2014 despite the bud being less developed compared to 2013.

Regeneration

In both years, treated twigs did not show severe sign of damage at a glance (Fig. 5). However, most of those shoots suffered die back with -12°C inflicting most fatality. Nevertheless, all treated plants survived freezing temperatures and were able to regenerate. The apical dominance in most of the treated plants was severely compromised irrespective of origin. This resulted to more shoots sprouting from either the base of the plant or from the lateral buds of the affected shoots. More shoots sprouted in Italian ecotype than the German ecotype (Fig. 6) and in the following order irrespective of origin: -12°C ≥ -6°C > 5°C. We had only regeneration plants in 2013 which were treated at -12°C of which were comparable to control in height.

Fig. 5. Morphological outlook of a German(A and B) and an Italian (C) ecotype twigs after frost treatment with -12°C in April 2013 and April 2014.

Fig. 6. Morphological outlook of German and Italian ecotype in September 2014 (five months of regeneration) following late frost (-12°C) treatment in April 2014.

DISCUSSION

In a common garden, ecotypes from higher altitude and or latitude are expected to be harder than those from low altitude or latitude since they are able to tolerate lower temperature of origin (Taschler and Neuner 2004). Imperatively plants originating from higher altitude or latitude ought to have lower electrolyte leakage in spring due to their delayed bud sprouting. When we consider the two German ecotypes (BB and RPF) evaluated for late frost damage in 2013, these expectations were not conformed as their ion leakage was similar. This could be explained by one reason: their origins' climatic conditions in spring are not much different to have had a considerable influence on their late frost hardiness (Table 1). This could be supported by their similarity in phenological development at the time of late frost experiment (Fig. 1). It is important to note that in spring, developing plants' tissues are less hardy than in winter and therefore prone to have high relative electrolyte leakage (REL) from expanding leaves' tissues (Taschler and Neuner 2004). Hence the high REL value even from the control plants. Hypothetically, the high REL values obtained in this experiment does not imply lethality as supported by the ability of regeneration plants to grow after exposure to the lowest temperature (Fig. 6). When we consider the German (BB) and the Italian (ITA) ecotype, evaluated the following year (2014), the influence of either altitude or latitude on REL was apparent. Contrariwise, the influence of altitude, although seemingly apparent, was excluded since it did not affect REL between the German ecotypes. The influence of latitude on REL could be explained indirectly on phenology. Since the Italian ecotype is originating from lower latitude its chilling requirement or its thermal requirement to bud break could be low as elucidated for apricot (Campoy et al. 2011). Consequently, it sprouted earlier and advanced its bud phenology faster than the German ecotype BB. Its fast expanding leaves were hence vulnerable to late frost damage as expounded by literature (Wanjiku and Bohne 2016). Expanding leaves have been demonstrated by literature to have a tendency to leak more ions due to their un-lignified leaves' cell wall (Taschler et al. 2004; Wanjiku and Bohne 2016) This was also demonstrated in these experiments where the German BB in 2014 when unstressed had higher REL than the BB in 2013 (Fig. 3). Similarly, the advanced leaf phenology of the Italian ecotype had higher ion leakage compared to that of German ecotype in 2014 (Fig. 4) late frost experiment even when the plants were not frozen (control).

Carbohydrates reserves are important carbon pool for growth and protection (Morin et al. 2007). When unstressed, the German ecotypes BB and RPF did not significantly differ from each other. However, when they were treated with low temperature (-6°C and -12°C), RPF significantly increased its glucose and sucrose concentration compared to BB. The increase in these two sugars seemed to a play a very minor role in constraining damage (Fig. 4). The failure of these two German ecotypes (especially BB) to increase their soluble carbohydrates, though speculative, could be as result of unavailable reserves. Most probably the carbohydrates reserves had been used up for sprouting as demonstrated by literature (Mohamed et al. 2012). Proline in these two ecotypes did not seem to follow any trend with temperature. For BB, it increased at -12 °C while for RPF it decreased. The reason for this opposing trend is not clear. Proline has been shown to accumulate with stress to alleviate or avoid loss of activity of many enzymes (Chen et al. 2014; Lei et al. 2006). In spite of its role, there is little information concerning latitudinal and altitudinal inclinations available.

Comparing the German (BB) and Italian ecotype, when untreated they did not differ in GFS and proline. The starch concentration of the Italian ecotype was low. This difference could be explained by the fact that the Italian plants were more advanced in sprouting than German (BB) ecotype and hence the starch concentration could have been hydrolysed to support new growth. Frost treatments had profound effects only at -12°C. The German (BB) ecotype increased its glucose and fructose concentrations, while the Italian ecotype increased its glucose, fructose, and sucrose and starch concentration. These increases could have helped the plants to withstand, some extent, lower temperature at -12°C as they maintained similar REL with that of -6°C (Fig.

4). This implies that the sugars increases were not sufficient to contain the late frost damage. The plants lost most of their twigs as can be seen from the regeneration plants (Fig. 6). The Italian ecotype starch increase (-12°C) may not be due to frost per se but could be related to a few plants, which by chance, had high starch concentration as can be supported with high standard deviation. It could be that the leaves that emerged early were photosynthetically active and efficient than those that emerged later as supported by literature (Lieth and Pasian 1990) although other photosynthetic intrinsic properties could influence. The evaluated German and Italian ecotypes differed in fructose and sucrose which could be speculated in terms of leaves developmental stage, where the Italian ecotype could be already photosynthesizing.

In the year 2014, proline concentration in both ecotypes (German and Italian) was not significantly influenced by late frost treatments as only a tendency to increase was observed (Table 3). This was similarly found with *Corylus avellana* late frost experiment (Wanjiku and Bohne 2016). Proline on these two German ecotypes was higher than that of Italian ecotype. This could be due to its speculative role in breaking bud dormancy and supplying energy as well as safeguarding enzymatic integrity during bud break (Chen et al. 2014; Walton et al. 1998) where the Italian ecotype was beyond this phenological stage.

The dissimilarities in German (BB) ecotype in 2013 and 2014 in some biomarkers concentration could be attributed to phenological stage, age, and genetics composition of the mother plants as reported by literature investigating genotypes (Aslamarz et al. 2011). Although these plants were greatly affected by the late frost treatments, they were able to regenerate by sprouting more shoots from the crown and by the end of the growing season (autumn), the Italian ecotype had attained a similar height as the dead leader shoot. The German ecotype's regenerated shoots were also proximal in length to the damaged leader shoot. The Italian ecotype was taller than the German ecotype.

Conclusion

Low temperature could induce frost damage causing destruction of plants at developmental stages. From the results above, frost damage could partially be related to the level of relative electrolyte leakage. This is because REL has proved to change with phenological stage and not exclusively with temperature and the level of damage. The biochemical changes due to low temperature in these experiments, seems to play a minor cryoprotective role against late frost damage in *Prunus spinosa*. Certainly, there could be other cryoprotective biomarkers involved other than the ones analyzed in this study. This could be explored further. The insignificant differences between German ecotypes could be an indication of low local adaptation or high plasticity to wide ecological range as alluded by literature. This suggests that they could be substituted for another in restoration of landscapes. However, the Italian behavior to sprout earlier could be maladapted for use in Germany particularly area with late spring temperatures in as it might frequently be frost damaged. Nevertheless, with increasing global warming, it might cautiously be utilized for its high growth rate and high ability to regrow even after frost damage.

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