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Salinity Tolerance in *Fraxinus angustifolia* Vahl.: Seed Emergence in Field and Germination Trials

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Abstract: The effect of salinity on seed germination/emergence in narrow-leaved ash (*Fraxinus angustifolia*) was studied both under field and laboratory conditions, in order to detect critical values to NaCl exposure. Research Highlights: Novel statistical methods in germination ecology has been applied (i) to determine the effects of chilling length and salinity (up to 150 mM NaCl) on *Fraxinus angustifolia* subsp. *oxycarpa* seed emergence, and (ii) to estimate threshold limits treating germination response to salinity as a biomarker. Background and Objectives: Salinity cut values at germination stage had relevant interest for conservation and restoration aims of Mediterranean floodplain forests in coastal areas subjected to salt spray exposure and/or saline water introgression. Results: Salinity linearly decreased germination/emergence both in the field and laboratory tests. Absence of germination was observed at 60 mM NaCl in the field (70–84 mM NaCl depending on interpolation model) and at 150 mM NaCl for 4-week (but not for 24-week) chilling. At 50 mM NaCl, germination percentage was 50% (or 80%) of control for 4-week (or 24-week) chilling. Critical values for salinity were estimated between freshwater and 50 (75) mM NaCl for 4-week (24-week) chilling by Bayesian analysis. After 7-week freshwater recovery, critical cut-off values included all tested salinity levels up to 150 mM NaCl, indicating a marked resumption of seedling emergence. Conclusions: *Fraxinus angustifolia* is able to germinate at low salinity and to tolerate temporarily moderate salinity conditions for about two months. Prolonged chilling widened salinity tolerance.

Keywords: Mediterranean wetland; NaCl salinity; *Fraxinus angustifolia*; seed germination

1. Introduction

Fraxinus angustifolia Vahl. (narrow-leaved ash) is a typical tree of hygrophilous mixed-hardwood forests of seasonally inundated wetlands along rivers and alluvial plains [1,2]. This tree species has been usefully employed in remediation [3,4] and urban greening programs [5–7] for its high ability to resist waterlogging [8], adverse conditions of soil contamination and for its moderate tolerance to salinity at seedling and tree stage [9–11], but nevertheless, in our knowledge, information on seed germination behavior under salinity conditions is still lacking.

Floodplain ashwoods are included in "interest" (Habitat 91F0—Riparian mixed forests along the great rivers) or "priority" (Habitat 91E0*—Alluvial forests) Natura 2000 lists under the European Union (EU) Habitats Directive (Annex I 92/43/EEC [12]), both relevant for conservation of vulnerable temporary water ecosystems in Mediterranean basin [13,14]. In this region and despite conservation efforts, contraction and degradation risk of wetlands and swamps are still ongoing processes, accounting more than 50% loss since 1900, with 10% decrease in recent times, from 1975 to 2005 [15–18]. In Europe, salinization affects 3.8 million ha [19,20], with a particularly severe wide spread of soil salinity along the coastlines, particularly in the Mediterranean [21], where further decline was

projected for the mid- (2050) and late (2100) century [22]. The global conservation awareness for these ecosystems—which nowadays resulted in a protected status under the international biodiversity-related multilateral environmental agreements [23] for more than one third of swamps, flooded forests and coastal wetlands—might not be sufficient to prevent degradation risks arisen from interactions with large-scale processes, land-use changes and agricultural land-use intensification. Consequently, undertaking practice and policy actions to prevent wetland degradation and loss remains a focus of international processes, such as the Convention on Biological Diversity (CBD) Aichi Targets and the United Nations (UN) Sustainable Development Goals (SDGs). In coastal floodplains, human pressure, altered water flows, contaminants from internal urban and agricultural lands, and increased soil salinity [24–26] were particularly relevant, and science-based management options should be urgent [27] to face adverse climate-related impacts exacerbated by increasing human-induced pressures [28–30].

F. angustifolia coastal alluvial forests were mainly judged under 'unfavorable' (habitat 91F0) or 'inadeguate' (habitat 91E0*) states, following the 2007–2012 EU Habitat Directive Art. 17 assessment [31]. The major threats to this species were currently considered to be high fragmentation with connectivity loss among sites [32,33], potential susceptibility to diseases (e.g., ash dieback by *Hymenoscyphus fraxineus* [34]), changes in water regime, availability and quality due to pollution, salinity [35,36] and saltwater intrusion [20]. In ash species, the effect of salinity could also be increased by the interaction with several other environmental conditions, such as radiation [37], drought [38] and waterlogging [39].

As ensuring natural regeneration of mixed-species forests is a highly recommended silvicultural practice for the management of ecologically 'natural' forests [40] also in marginal environments [41], this paper was aimed at evaluating seed germination/emergence under increasing salinity conditions (in field and laboratory), to estimate NaCl tolerance threshold values at this life stage in *F. angustifolia*. Moreover, novel statistical methods for germination ecology (based on survival analysis and hierarchical Bayesian analysis) have been applied to infer cut off values for salinity threshold.

2. Materials and Methods

2.1. Germination in Natural Environment

Within the coastal hygrophilous mixed-hardwood forest (43.69° N, 10.29° E; Natural Reserve "Lame di fuori"—a complex of 655 ha of swamps and marshes [42–45] in San Rossore Park, Tuscany, Italy), three sites were sampled at different distances from the seaside, which differed in soil salinity (Table 1). Sampled sites were dominated by *F. angustifolia* subsp. *oxycarpa* both in number (n_{tree} = 54) and basal area (BA = 71%), associated with field elm (*Ulmus minor*, n_{tree} = 38%; BA = 25%), black alder (*Alnus glutinosa*, n_{tree} = 1%, BA = 2%), common hawthorn and common pear (together, n_{tree} = 7%, BA = 1%). Average (±SD, standard deviation) for forest stand traits were: 18 (±1) m dominant height, 18 (±1) cm breast tree diameter, 51 (±1) years of age, 1104 (±267) trees ha^{-1} and 35 (±7) m^2 ha^{-1} basal area. Narrow-leaved ash seedlings at cotiledon stage were counted at the end of June 2013 within 3 randomly placed (origin and direction) transects (40 m × 1 m) per site, not crossing each other. Ten soil cores per transect (0–20 cm, litter layer removed, spaced every 4 m) were sampled, air-dried and 2 mm sieved before laboratory analysis at 25 °C. Soil salinity determined by ECe (electrical conductivity of soil saturation extract [46]), then NaCl concentration derived by $NaCl_{(mM)} = 9.5 \times ECe_{(dS/m)} - 1.1327$ calibration equation. Soil samples had a sandy texture and pH (soil:water, 1:5 [47]) of 6.7 (coefficient of variation, CV = 10%).

All statistical analyses were performed in R environment [48]. Normality of data distribution was tested (Shapiro-Wilk W-test [49] by shapiro.test() R function [50]). As most of the variables were not normally distributed, comparisons among groups were performed by non-parametric Wilcoxon rank-sum test by the wilcox.test() R function [51], while mean, standard error (SE) and 95% confidence interval range (95% CI) were calculated by bootstrapping applying boot.ci() function in '*boot*' R package [52] along with the adjusted bootstrap percentile (BCa) method [53].

2.2. Germination Trials

A bulk of narrow-leaved ash samaras from San Rossore Park was tested for seed vitality by the TZ (tetrazolium) test [54,55]. Embryo development was assessed by embryo length to seed length ratio (E:S ratio) on 50 seeds. Samaras (intact pericarp) were manually graded to remove aborted seeds or empty samaras, soaked in water overnight, then moist-stratified at 4 °C (*chilling* effect, C) for four (C_4) or 24 (C_{24}) weeks. Germination took place at 15 °C/25 °C (16 h dark/ 8 h light) for a period of 8 weeks, testing a range of salinity up to 150 mM NaCl (*nacl* effect, N_x where x is NaCl in mM). Freshwater (control, N_1) had naturally dissolved 1 mM NaCl together with other cations (1.5 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.1 mM K^+). The salinity levels were obtained by adding NaCl to freshwater up to the concentration (in mM) indicated by N_x subscript, that is N_{10}, N_{50} and N_{150} for 4-week chilling (C_4) and N_{50}, N_{75}, N_{100} and N_{150} for 24-week chilling (C_{24}), respectively. About 200 samaras per treatment (224) were distributed in 8 germination boxes (Ø = 15 cm [56]). Germination was assessed weekly as 'visible germination' (i.e., radicle protrusion > 1 cm [57]). At the end of the germination test, C_4 samaras underwent a recovery germination test; samaras were rinsed three times for 10 min in flowing water, placed in N_1, then followed for further 7 weeks.

Table 1. Descriptive statistics for soil salinity (0–20 cm) and number of *Fraxinus angustifolia* seedlings in "Lame di fuori" sampling sites (S1–S3). Distance from seaside (m); N = number of samples (soil) or transects (seedlings). W = *p*-value for Shapiro–Wilk normality test. ECe (electrical conductivity of soil saturation extract) = soil ECEe min–max range in dS/m. % = fraction (in percent) on the total of saline (ECe ≥ 4 dS/m) soil samples.

Site	(m)	N	Minimun–Maximum	Mean (SE)		95% CI	W	ECe	%
				soil (mM NaCl)					
S1	890	30	32–95	55 (2.6)	a[1]	50–61	0.345	3.1–10.2	96
S2	1210	30	3–102	38 (5.6)	b	28–50	0.008	4.1–11.2	45
S3	1650	30	18–41	27 (0.8)	b	25–29	0.009	2.7–4.4	7
Total	1250	90	3–102	40 (2.4)		35–45	0.001	2.7–11.2	48
				seedlings (ha^{-1})					
S1		3	0–500	167 (96)	b[1]	0–333	0.001		
S2		3	0–1500	667 (226)	ab	167–1000	0.415		
S3		3	0–2500	1250 (307)	a	583–1750	0.751		
Total		9	0–2500	694 (168)		389–1028	0.010		

[1] Wilcoxon test (different letters for *p* < 0.05).

2.3. Statistical Analysis

Seed germination was assessed by survival analysis [58], where the response in terms of germination was defined as time-to-event [59,60]. Respect to the classic approach of calculating germination capacity and other germination indexes, this method offered the advantage of a proper statistical handling for time-repeated measures (namely the weekly counts of germinated seeds) together with an evaluation of treatment effects over the entire trial period [61]. An event was defined as the change from ungerminated (value = 0) to germinated status (value = 1); only viable seeds (assessed by cut test) were included in the counts. For each samara, the response variable T 'time until an event occurred' was counted in weeks since the beginning of the trial (germination- or recovery-test, respectively). The probability of germination per unit of time, $f(t)$, was calculated by T probability density function (PDF); while the probability of changing status (germinating) within time t $(T \leq t)$, $F(t)$, was given by the cumulative distribution function (CDF). Here, the survival function, $S(t) = 1 - F(t)$, returned the probability of not germinating within time t, that is the probability that a seed had to remain in its original (ungerminated) state for a time longer than t $(T > t)$.

The hazard rate of germinating, h, was defined as the probability that an ungerminated seed (until the beginning of a time interval) had to germinate in the successive time interval (i.e., the

conditional rate of germinating). It followed that the hazard function, $h(t) = f(t)/S(t)$, returned the rate at which events occur for those seeds, that have not yet germinated at time t. The integral of $h(t)$ over time gave the cumulative hazard rate function, $H(t)$; the accumulated risk that a seed would face up to time t. Non-parametric methods were used for estimating both survival function $\hat{S}(t)$ (Kaplan and Meier method, KM [62]) and cumulative hazard rate function $\hat{H}(t)$ (Nelson–Aalen method, NA [63–65]). These non-parametric estimators did not assume any underlying distribution and they returned non-parametric maximum likelihood estimators (NPMLE) for survival and hazard functions, respectively, in right-censored data [66]. A 95% confidence interval (CI) for $\hat{S}(t)$ was obtained by minimizing the likelihood function [67] within '*km.ci*' R package [68], then it was derived for $\hat{H}(t)$ by $-\log \hat{S}(t)$ of 95% CI values. Hazard ratio (HR) could be interpreted as a relative measure of effects, as it compared survival between two treatments by $S_0(t) = S_1(t)^{HR}$, where S_0 and S_1 are the survival probabilities in control and in an alternative treatment, respectively. Therefore, HR = 1 indicated no difference between treatments; HR values greater (or lower) than 1 indicated a higher (or lower) hazard of germinating in the control respect to the alternative treatment [69,70].

Chilling, salinity and their interaction effects on germination were tested by semi-parametric Cox proportional hazards regression model (Cox PH [71]), applying coxph() function in '*survival*' R package [72]. The Cox PH model had no assumption about the shape of the hazard function, but it assumed proportional hazards rates among treatments, no time change in tested variables, lack of influential observations and linearity. Assumptions were verified by plotting scaled Schoenfeld residuals [73] against time [74]. The significance of model terms (*nacl*, *chilling*, *nacl × chilling*) was assessed by Wald test; differences among treatment levels were compared by Peto-Peto-Prentice generalized Wilcoxon test—this latter did not require a consistent hazard ratio over time, but only that $H(t)$ curves did not cross [60].

Backward stepwise regression using drop1() R function and likelihood ratio (LRT) were used for model reduction. Differences in goodness of fit for each model were then performed by log-likelihood, Akaike information criterion (AIC) and Bayesian information criterion (BIC), using logLik() and stepAIC() functions in '*MASS*' R package, respectively [75]. In order to select the best model (most parsimonious; the simpler model with a high explanatory predictive power), the lowest (with difference exceeding 2) AIC (or BIC) and the highest likelihood values (LLV) were used. Cut off values for salinity in germination trials were determined fitting biomarker threshold models by '*bhm*' R package version 1.15 [76,77], treating germination/emergence response to salinity (*nacl* levels) as a biomarker. The aim of this hierarchical Bayesian method was to identify within the dataset a range of salinity (and its credible intervals), which were most likely to give a similar response to the best treatment in terms of germination probability. Variance inflation factor (VIF = $1/(1-r^2_{adj})$ was calculated to detect multi-collinearity amongst the predictor variables. VIF lower bound of 1 indicated no relationship between predictors; for VIF values till 2.5 collinearity was not an issue, becoming of concern above 2.5 (or 5) [69,70].

3. Results

3.1. Seed Germination in Natural Environment

Soil salinity had an average ECe of 4.1 (±0.3 SE) dS m^{-1} and 40 (±2.4 SE) mM NaCl with a wide variability in S1 and S2 sites, both including values higher than 10 dS m^{-1} (Table 1). In transects, newly germinated seedlings were found up to 53 mM NaCl soil salinity, and no seedling was found above 60 mM NaCl. The relationship between seedling density and soil salinity could be explained by linear (r^2_{adj} = 0.466, $p < 0.026$, n transects = 9) or exponential (r^2_{adj} = 0.688, $p < 0.003$, n = 9) functions. The ratio between intercept (1532.7 ha^{-1}) and slope (-22.0 ha^{-1}/mM NaCl) in linear interpolation estimated the absence of seedlings at 70 mM NaCl. Here, by leave-one-out validation procedure (i.e., removing one transect per time from the analysis), this estimate ranged between 65 and 73 mM NaCl and it had a bootstrapped 95% confidence interval range of 67–71 mM NaCl. An exponential model estimated the absence of seedlings at higher salinity (84 mM NaCl, Figure 1).

3.2. Seed Viability and Embryo Development

Seed lot had 70% viable, 22% non-viable, and 8% unclassified seeds (TZ test). Normality was observed only for embryo length in non-viable seeds and E:S ratio in viable seeds (W test, $p > 0.14$; $p > 0.32$, respectively). The two viability classes did not differ for seed and embryo development (Wilcoxon test: seed length, $p > 0.14$; embryo length, $p > 0.63$; E:S ratio, $p > 0.77$). Overall, seed and embryo length had an average of 17 mm (±0.27 SE; min–max: 10–22) and 12 mm (±0.23 SE; min–max: 6–15), respectively, with E:S ratio of 0.7 (±0.01 SE; min–max: 0.4–0.9). Prediction of embryo length by seed length was poor, even if significant ($r^2_{adj} < 0.14$, $p < 0.004$). E:S ratio was more influenced by embryo length increase (slope = 0.038, $r^2_{adj} = 0.39$, $r = +0.63$, $p < 0.000$), than by seed length decrease (slope = −0.023, $r^2_{adj} = 0.19$; $r = −0.45$, $p < 0.000$).
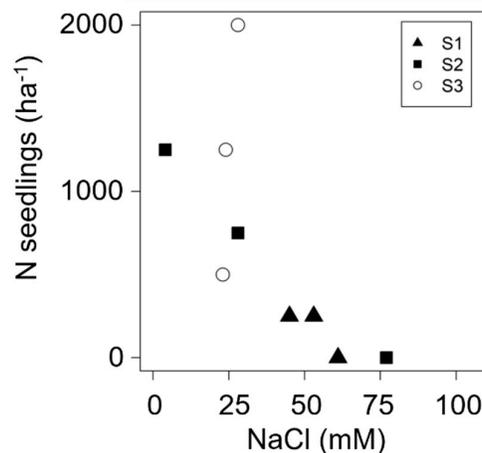

Figure 1. Salinity and seedling density at the three sites (S1–S3, see Table 1) in "Lame di fuori".

3.3. Chilling and Salinity Effect in Germination

After 8 weeks, germination was higher in C_{24} than in C_4 ($\hat{F}$, 15% *vs.* 9%, $p < 0.01$; Figure 2a). Longer chilling treatment showed a marked higher germination hazard rate in the first week, while, thereafter, similar hazard rates indicated comparable germination between chilling treatments (Figure 2b). Final germination percentage ($\hat{F}$) decreased with salinity, ranging from 0% to 12% in C_4, and from 6% to 23% in C_{24} (Figure 3a,b). Highest and promptest germination was observed in C_{24} at low salinity (≤50 mM NaCl). Complete absence of germination occurred only for C_4 at 150 mM NaCl. C_{24} strongly germinated during the first-week, when hazard rate was more than 10 times higher with respect to what observed in C_4 or at any other time in the trial (Figures 2 and 4a,b). C_{24} first week germination was linearly and positively related to final $\hat{F}$ values (Figure 5a), and it had a marked effect on final $\hat{F}$ value, particularly at high salinity (up to 89% of $\hat{F}$ at 150 mM NaCl), representing almost all observed events, while at low salinity (≤50 mM NaCl) it contributed only 53% of $\hat{F}$ (Figure 5a inset).

By contrast, the quicker chilling length, C_4, showed germination during the first week only in control (hazard rate, $h = 1.5\%$), and about 3 to 4 weeks were needed to reach at least 50% of $\hat{F}$ for low salinity levels up to 50 mM NaCl. Apart from this initial difference, since the second week forward hazard rates did not significantly differ between chilling treatments at any salinity level (paired Wilcoxon test, $p > 0.43$), showing a ratio not different from unit both for control (N_1, $h_{C_4}/h_{C_{24}}$ ± SE: 1.04 ± 0.38, $p > 0.43$) and common salinity levels, obtained by pooling N_1, N_{50} and N_{150} together ($h_{C_4}/h_{C_{24}}$ ± SE: 1.27 ± 0.33, $p > 0.59$).

(**a**)

(**b**)
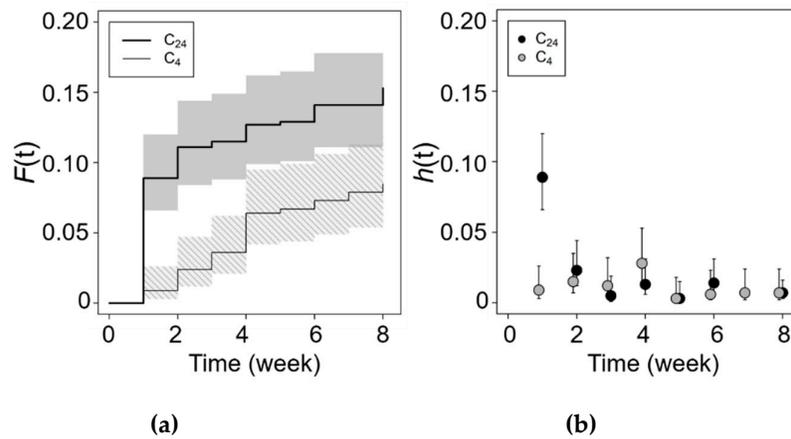
Figure 2. Germination test; chilling treatments (C$_{24}$ and C$_4$) averaged over common salinity levels (N$_1$, N$_{50}$ and N$_{150}$). (**a**) germination probability, *F*(t); (**b**) hazard rate, *h*(t). Mean ± 95% CI bands or bars calculated by boot.ci() function in *'boot'* R package. Values of *h*(*t*) = 0 (no germination) were not reported (e.g., C$_{24}$ at week 7).

(**a**)

(**b**)

(**c**)
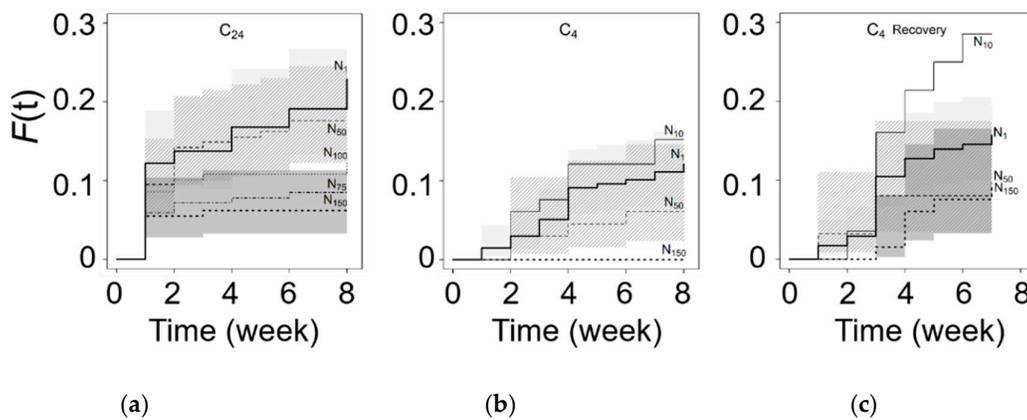
Figure 3. Cumulative germination probability, *F*(t), for different salinity (Nx; x = NaCl in mM) during germination (**a**, **b**) and recovery (**c**). Mean ± 95% confidence interval (CI) bands for N$_1$ (light gray), N$_{50}$ (diagonal stripes) and N$_{150}$ (dark gray). Same symbols in all plots.

(**a**)

(**b**)

(**c**)
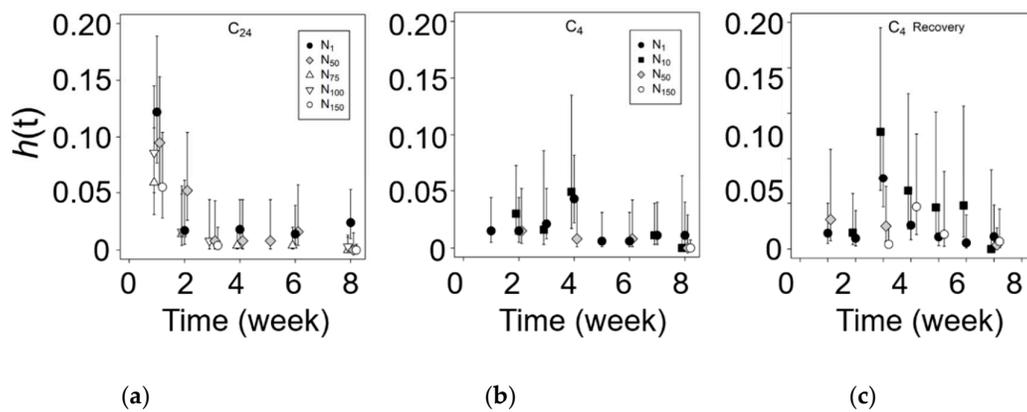
Figure 4. Hazard function, *h*(*t*), under different salinity during germination (**a**, **b**) and recovery (**c**). Mean ± 95% CI bars. Same symbols in all graphs. See Figure 2 for further methodological details.

| (**a**) | (**b**) |
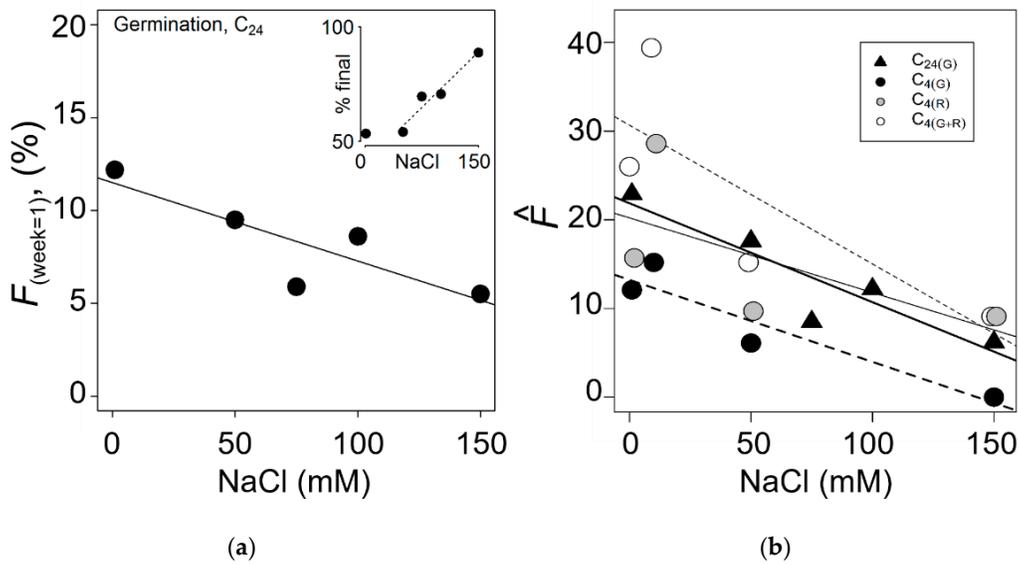
Figure 5. (a) Salinity and C_{24} first-week germination probability, $F_{(week=1)}$; $y = -0.04^{ns}x + 11.5^{**}$, $r^2_{adj} =$ 0.633, $p > 0.067$, $n = 5$. Inset: salinity and relative germination risk for 1st week relative to final germination (in percent), % final $= 100 \cdot F_{(week=1)}/\hat{F}$; for 50–150 mM NaCl salinity range, $y = 0.32^* x + 40.3^{**}$, $r^2_{adj} = 0.915$, $p < 0.029$, $n_{obs} = 4$. **(b)** Linear regression between salinity and $\hat{F}$ in the trials (G = germination, R = recovery): *solid bold*, $C_{24(G)}$, $r^2_{adj} = 0.77$, $p < 0.031^*$; *dashed*, C_4, $r^2_{adj} = 0.76$, $p < 0.034^*$; *solid*, $C_{4(R)}$, $r^2_{adj} = 0.37$, $p < 0.105^{ns}$; *densely dashed*, $C_{4(G+R)}$, $r^2_{adj} = 0.47$, $p < 0.197^{ns}$. * $= p < 0.05$, ** $= p < 0.01$.

Salinity (*nacl*) and chilling length (*chilling*) tested by Cox PH model were significant, without a significant interaction effect (Table 2). Hierarchical Bayesian model '*bhm*' and LRT analysis confirmed these results (Tables 2 and 3). In comparing models, interaction was not significant (as shown by *p*-value >0.05 for likelihood ratio and AIC values below 2). Moreover, several other statistics pointed out to the exclusion of interaction from model: *nacl × chilling* interaction vs. *nacl* VIF values well above the suggested 2.5 threshold [69], and BIC better goodness of fit for *nacl + chilling* model (Table 3). When individually considered, *nacl* contributed more than *chilling* to model goodness of fit (Table 3).

Table 2. Summary table for Cox proportional hazards (PH) regression model and cut off values for *nacl* biomarker threshold ('*bhm*') in germination (G) and recovery (R) trials. Cox proportional hazard assumption (PH *p*) rejected if $p < 0.05$ (in **bold**). Effects (Z_i) tested by Wald and '*bhm*' tests ($p < 0.05$, in **bold**). Cut off values for *nacl* biomarker threshold (in mM NaCl) by '*bhm*' analysis and their 95% CI (in brackets).

Trial	Z_i	PH p	Wald p^1	'*bhm*' p	Cut off Values (95% CI)
Complete dataset					
G	*nacl*	0.930	**0.016**	**0.011**	1–50
	chilling	**0.004**	**0.011**	**0.008**	(1-1) – (50-50)
	interaction	0.526	0.155	0.218	
1st week excluded					
G	*nacl*	0.559	**0.030**	**0.022**	1–50
	chilling	0.383	0.830	0.585	(1-1) – (50-50)
	interaction	0.904	0.639	0.644	
C_{24}					
G	*nacl*	**0.014**	**0.000**	**0.000**	1–75 (1-1) – (75-75)
C_4					

G	*nacl*	0.683	**0.009**	**0.005**	1–50 (1-1) – (50-50)
R	*nacl*	0.104	0.057	**0.021**	10–50 (1-10) – (50-150)
G + R	*nacl*	**0.010**	**0.001**	**0.000**	1–50 (1-1) – (10-150)

[1] $p < 0.01$ for Cox model (by likelihood ratio (LRT), Wald and logrank tests) in all trials except recovery (R: LRT, $p < 0.050^*$; Wald, $p < 0.057^{ns}$; logrank, $p < 0.052^{ns}$).

Table 3. Model selection (best models in **bold**) by log-likelihood value (LLV), Akaike information criterion (AIC), Bayesian information criterion (BIC); df, degree of freedom. Likelihood ratio (LRT) *p*-value listed following the order of terms in model formula (significant terms in **bold**, $p < 0.05$).

Model [1]	df	LLV [2]	AIC	BIC	LRT *p*-Value
Complete dataset (Germination)					
nacl + chilling + nacl × chilling[1a]	3	**−906** *a*	1818 [3]	1833 [3]	**0.001, 0.011,** 0.092
nacl + chilling	2	**−907** *a*	1819 [4]	1829 [4]	**0.000, 0.000**
Nacl	1	−916 *b*	1833	1838	**0.000**
Chilling	1	−923 *d*	1848	1853	**0.047**
nacl × chilling	1	−922 c	1846	1850	**0.015**
None	0	−925 *e*	1850	1850	
1st week excluded (Germination)					
nacl + chilling + nacl × chilling[1b]	3	**−473** *a*	952 [3]	967 [3]	**0.003,** 0.830, 0.626
nacl + chilling	2	**−473** *a*	951 [4]	960 [4]	**0.000,** 0.605
nacl	1	**−473** *a*	**949**	**954**	0.000
chilling	1	−489 *c*	981	986	0.034
Nacl × chilling	1	−479 *b*	959	964	0.000
none	0	−492 *d*	983	983	
C_{24} *(Germination)*.					
nacl	1	**−609** *a*	**1220**	**1225**	0.000
none	0	−618 *b*	1236	1236	
C_4 *(Germination)*					
nacl	1	**−218** *a*	**437**	**442**	0.000
none	0	−215 *b*	451	451	
C_4 *(Recovery)*.					
nacl	1	**−317** *a*	**635.1**	**637.4**	0.037
none	0	−319 *b*	637.4	640.1	
C_4 *(Germination + Recovery)*					
Nacl	1	**−559** *a*	**1120**	**1125**	0.000
none	0	−567 *b*	1134	1134	

[1] VIF (variance inflation factor), [1a(1b)] nacl vs. chilling: 1.1 (1.2); nacl×chilling vs: nacl: 4.2 (4.0); chilling: 2.1 (2.2). [2] Best model selection by analysis of deviance; different letters for $p < 0.05$. [3] AIC (BIC) values after single term drop from model. *Complete dataset (Germination)*: *nacl × chilling*, 1819 (1829); *chilling*, 1823 (1833); *nacl*, 1823 (1838). *1st week excluded (Germination)* dataset: *nacl × chilling*, 951 (961); *chilling*, 950 (960); *nacl*, 959 (960). [4] AIC (BIC) values after single term drop from model. *Complete dataset (Germination)*: *chilling*, 1833 (1838); *nacl*, 1848 (1852). *1st week excluded (Germination)* dataset: *chilling*, 949 (954); *nacl*, 981 (986).

In the Cox PH model the assumption of proportional hazard held for *nacl* ($p > 0.93$) and interaction effect ($p > 0.53$), but not for *chilling* ($p < 0.004$), mainly because of the high hazard rate during the first week in C_{24} for any of the salinity levels (Figure 4a,b; Table 4). PH assumption

rejection for *chilling* suggested to test salinity effect under conditions that met such assumptions; either by excluding first week germination from data analysis, or by separately evaluating *nacl* effect within C_4 and C_{24}. The former analysis showed that, after the first week, hazard became proportional for all effects. In doing so, only *nacl* (but not *chilling* nor interaction) remained significant under Cox PH model ($p < 0.030$, Table 2). *'bhm'*, LRT, AIC and BIC analyses confirmed this result, indicating that most of the differences between chilling treatments were in the first week (Tables 2 and 3), as also shown by time trends for survival and hazard rate (Figure 2).

Table 4. Cumulative germination probability ($\hat{F}$) and cumulative hazard rate ($\hat{H}$) in percent along with their 95% CI range (in brackets). Differences between C_{24} and C_4 for the same salinity levels (*nacl*, in mM) in **bold** (Wilcoxon $p < 0.05$); within each trial different letters for Wilcoxon $p < 0.05$. RR (= $\hat{F}_{Nx}/\hat{F}_{N1}$) = relative risk respect to control.

chilling	*nacl*	$\hat{F}$ (CI range)	$\hat{H}$ (CI range)		RR
		Germination			
C_{24}	1	**22.9** (16.5–30.8)	**25.0** (17.1–35.9)	*a*	1
	50	**17.6** (12.3–24.5)	**18.7** (12.5–27.5)	*ab*	0.8
	75	8.5 (5.0–14.0)	8.7 (5.0–14.9)	*c*	0.4
	100	12.2 (7.8–18.7)	12.6 (7.7–20.4)	*bc*	0.5
	150	**6.2** (3.3–11.3)	**6.2** (3.2–19.1)	*c*	0.3
	1+50+150	**15.3** (12.2–19.0)	**16.1** (12.5–20.6)		
C_4	1	12.1 (8.3–17.4)	12.8 (8.5–19.0)	*a*	1
	10	15.2 (8.4–25.7)	16.0 (8.5–29.4)	*a*	1.3
	50	6.1 (2.4–14.6)	6.2 (2.4–15.7)	*a*	0.5
	150	0.0	0.0.	*b*	0
	1+50+150	8.5 (5.9–12.0)	8.8 (6.1–12.7)		
		Recovery			
C_4	1	15.7 (11.0–21.9)	16.7 (11.3–24.3)	*b*	1
	10	28.6 (18.4–41.5)	32.2 (19.1–52.3)	*a*	1.8
	50	9.7 (4.5–19.6)	10.0 (4.5–21.6)	*b*	0.6
	150	9.1 (4.2–18.5)	9.4 (4.2–20.3)	*b*	0.6
	1+50+150	13.0 (9.7–17.3)	13.4 (9.9–18.7)		
		Germination + Recovery			
C_4	1	26.0 (20.4–32.6)	29.6 (22.2–38.9)	*ab*	1
	10	39.4 (28.5–51.5)	48.0 (31.7–70.4)	*a*	1.5
	50	15.2 (8.4–25.7)	16.2 (8.6–29.5)	*bc*	0.6
	150	9.1 (4.2–18.4)	9.4 (4.2–20.3)	*c*	0.3
	1+50+150	20.4 (16.4–25.1)	22.5 (17.6–28.6)		

C_{24} showed germination also at 150 mM NaCl (6.2%), with cumulative germination curve, $F(t)$, statistically not significantly different from moderate–high salinity (N_{75}, $p > 0.44$; N_{100}, $p > 0.08$), but highly different from low salinity levels (N_{50}, $p < 0.003$; control, N_1, $p < 0.000$). At low salinity (N_1 and N_{50}) no difference in germination curves was observed between these two levels ($p > 0.29$), both showing higher germination respect to moderate and high salinities ($p < 0.023$, Table 4, Figure 3).

Salinity negatively influenced germination independently from chilling treatment, being *nacl* effect highly significant ($p < 0.010$) over and within each chilling level (Tables 2 and 3). Comparing the two chilling lengths (C_{24} vs. C_4), longer chilling resulted in an overall higher germination (generalized Wilcoxon test, $p < 0.004$), also confirmed within each salinity level (N_1, $p < 0.006$; N_{50}, $p < 0.020$; N_{150}, $p < 0.040$). The salinity levels (in common over chilling treatments) showed similar germination curves for N_1 and N_{50} ($p > 0.58$), both higher than N_{150} ($p < 0.000$). Within C_4, 150 mM NaCl failed to germinate and significantly ($p < 0.05$) differed from all the other salinity levels; while at low salinity (≤50 mM NaCl) no statistically significant difference was observed between *nacl* levels

(N$_1$ vs. N$_{10}$, $p > 0.52$; N$_1$ vs. N$_{50}$, $p > 0.17$; N$_{10}$ vs. N$_{50}$, $p > 0.09$; Table 4, Figure 3). Aggregating C$_{24}$ data in three salinity ranges of 50 mM NaCl–*low*, *moderate*, and *high*; (0, 50], (50, 100] and (100, 150]—germination in *low* salinity was higher ($p < 0.001$) with respect to each of the other two classes, being the last two ones not statistically different ($p > 0.15$). Applying *'bhm'* model in order to determine cut off values for conditions that could be interpreted as homogenous, the range between 1 mM to 50 mM NaCl was inferred for (i) all data pooled together and (ii) C$_4$, while C$_{24}$ had a wider salinity range from 1 to 75 mM NaCl (Table 2).

3.4. Recovery from Salt Stress

In recovery, C$_4$ final germination was highest under 10 mM NaCl ($\hat{F}$ = 29%) followed by control (16%), 50 mM NaCl (10%) and 150 mM NaCl (Table 4, Figure 3). Samaras in high salinity (N$_{150}$) did not germinate until the third week in freshwater, with a peak of hazard rate in the fourth week (h = 4.6%). Salinity was not significant (but close to critical value) in survival analysis, by contrast with *'bhm'* ($p < 0.021$, Table 2), AIC and BIC (chi square $p < 0.037$, Table 3) analysis, even if AIC and BIC values were only slightly higher than the threshold value of 2 (AIC, 2.3; BIC, 2.7).

Salinity at 10 mM NaCl had a higher germination probability curve respect to any other *nacl* treatment (Wilcoxon test, Figure 3c) without statistically significant difference among these latter ones (i.e., 1, 50 and 150 mM NaCl, Table 4). Cut off values for salinity during recovery (determined by *'bhm'* and including confidence intervals) extended over the full salinity range tested—from control up to 150 mM NaCl.

An overall evaluation, which included the 15 weeks of germination and recovery tests (G+R), again showed the highly significant effect of salinity (by Cox PH model, *'bhm'*, AIC, BIC and LRT; Tables 2 and 3). Highest germination ($\hat{F}$) was achieved at 10 mM NaCl (N$_{10}$, 39%), followed by control (N$_1$, 26%), low (N$_{50}$, 15%) and high (N$_{150}$, 9%) salinity (Table 4). Overall, salinity at 10 mM NaCl did not differ ($p < 0.05$) from control, while it was statistically higher from N$_{50}$ and N$_{150}$ salinity levels. Control $\hat{F}$ was higher only respect to high salinity (N$_{150}$), while N$_{50}$ and N$_{150}$ did not differ (Table 4). The cut off values inferred by *'bhm'* did not substantially deviate from the results obtained by the recovery test. Since germination-test beginning, two peaks of germination had been observed in control and 10 mM NaCl treatment, namely at the 4th and 11th weeks (i.e., the 3rd week of recovery, Figure 4b,c).

The negative trend between salinity and $\hat{F}$ had comparable slopes among trials. It should be noted that relationship between salinity and $\hat{F}$ was significant only for C$_{24}$ and C$_4$ germination (r^2_{adj} > 0.77; 0.76, respectively, both $p < 0.05$), but not for recovery (r^2_{adj} = 0.37, $p > 0.10$), nor germination + recovery (r^2_{adj} = 0.47, $p > 0.20$) trials. In all salinity levels, final $\hat{F}$ for C$_{4(G+R)}$ was greater than C$_4$ and similar to C$_{24}$ (as inferred by 95% CI range, Table 4). In control (intercept in Figure 5b) C$_{24}$ > C$_4$ and C$_{24} \approx$ C$_{4(R)}$ < C$_{4(G+R)}$, highlighting the effects of chilling and recovery on germination.

4. Discussion

4.1. Chilling Effect

The comparison of the two chilling lengths confirmed that northern Tuscany (Italy) *F. angustifolia* population had an intermediate physiological seed dormancy (PD, following the Baskin and Baskin definition [78,79]), and took advantage of a prolonged chilling period. The time required to break germination has a central role not only for physiological dormancy classification, but also to prevent freshly matured seed from germinating under otherwise favourable conditions, such as under warm autumn days in Mediterranean climate [80,81]. The two chilling lengths tested in this study were placed to either side of recommended values (4–26 weeks) for *F. angustifolia* [79] and intermediate PD species (1–6 months [78]). In control, 24-week long chilling resulted in nearly double germination probability (23%) with respect to 4-week chilling (12%), and this latter treatment needed almost double the period of time (15 instead to 8 weeks) to reach similar values to the former. Seed dormancy was mainly attributed to seed coat, as *F. angustifolia* isolated embryos could grow and develop into seedling [82]. The observed low germination, even in freshwater conditions, could partly be

explained by the choice of not having applied two dormancy-breaking procedures, that is (i) after-ripening freshly harvested mature seeds in a relatively dry state (effective also for this species [83]) and (ii) to remove pericarp before dormancy-release treatments [79,84]. Both choices were justified by the purpose of investigating germination response to chilling and salinity in conditions similar to those experienced by seeds after dispersal in the natural environment.

F. angustifolia populations showed a decrease of dormancy from northern to southern Italian provenances [85], without chilling requirement in Sicily [83]. At the northern geographical range (41°–43° Lat. N), lack of cold stratification resulted in low germination percentage (below 25% after 7–8 weeks) for Spanish [86], French [87], and Turkish [88] provenances, suggesting that origin site temperature or other environmental cues might play a role in dormancy levels [89,90]. Assuming a linear increase germination with chilling length for our seed lot in freshwater conditions, germination probability would be reduced to $\hat{F}$ = 10% without chilling (intercept) with a germination increase of 0.54% per chilling week (slope), not so much lower than what was inferred from the Turkish provenances dataset (0.8%–1.4% per week) [88].

Chilling promotes germination, as soon as temperature (and light) conditions returned favourable (as evidenced by C_{24} high germination rate during the first-week, Figure 1), allowing emergence also in the sub-optimal conditions given by salinity. Most of the chilling effect was detected in the first week. Excluding first-week emergence from data analysis both Bayesian and survival analysis showed comparable germination between the two chilling lengths (Table 2). A week of high germination rate could result in an effective mechanism to escape the negative effect of salinity for a higher fraction of seeds. In several plant species (salt-intolerant glycophytes included), cold stratification under freshwater conditions induced emergence under saline conditions [91]. Cold stratification might induce several physiological changes involved in germination under salty conditions, such as higher seed imbibition [92,93], changes in enzyme kinetics or activities [94,95], and/or storage protein degradation [96,97].

4.2. Salinity Effect in Laboratory and in the Field

Salinity reduced seed germination in plants [98–101], even in halophytes [102–104], delaying or preventing germination beyond tolerance limits [105]. Although the issue of critical values' determination for salinity is complex and controversial—due to the diversity of saline environments and responses induced by salts, such as: osmotic and oxidative stresses, ion-toxicity and/or nutritional disorder [106]—generalized relationships between germination curves and salinity were suggested. Following the Läuchli and Grattan classification [107], a delay in germination onset (without differences in rate and thus in final germination percentage) sorted 'moderate' from 'low' salinity levels, while a further delay along with significant final germination percent reduction characterized 'high' salinity levels. This scheme might not be confirmed by C_{24} results (which did not show any delay in germination onset; or at least it was limited to 1 week, where only a reduction of final germination with 75 mM NaCl and beyond was observed, Figure 3a). On the other hand, a 'moderate' salinity effect up to 50 mM NaCl resulted in C_4 treatment, suggesting the important role of chilling to overcome dormancy and saline stress (Figure 3b).

In our dataset, seed germination was progressively reduced under salinity conditions and marked reduction of seed germination over 50 (75) mM NaCl was highlighted both under laboratory (Cox PH and hierarchical Bayesian analysis) and natural environment conditions (where according to the interpolation equations the absence of seedlings should be estimated in the range from 65 to 84 mM NaCl, Figure 1), indicating that this species might complete germination under 'low' to 'moderate' salt stress [108]. Even if in this study seedling sampling was performed over a short time-period, the comparison of seed germination response to salinity in natural environment and germination tests resulted useful to infer tolerance levels over a wide range of environmental conditions. It has often been pointed out that salt tolerance of plants is difficult to quantify in natural environment, because it may vary with environmental conditions, plant life stage, and genotype [109,110]. Sampling newly germinated seedling in the forest surely had the advantage to return results based on effective environmental conditions, but nevertheless such trials had several *cons* in

identifying cause-effect relationships (e.g., [111]). Time and space variability in abiotic or biotic factors and conditions (apart from salinity) might strongly influence results; here, site vicinity (below 1 km), similar soil and stand conditions could at least in part minimize this problem. On the other hand, estimates obtained by standard laboratory germination tests may not reflect seedling emergence in the field [112], and the extrapolation of monosaline response (such as NaCl) to field salinity conditions might be only speculative [113]. The critical values found in our study were similar to those detected in *F. rhynchophylla* [114] and slightly lower than what was reported for *F. chinensis*, which is able to retain 60% of control germination at 100 mM NaCl [115]. In *F. angustifolia* embryogenic callus culture growth, osmotic and ion-toxicity were evidenced at 100 mM NaCl [116].

4.3. Recovery

A marked and positive influence of the period in freshwater was observed, which reduced, at least in part, the negative effects of salinity (even if Cox PH results applied to the entire trial, i.e., germination + recovery, should be interpreted with prudence, as salinity changed between germination and recovery trial for most treatments, infringing assumptions). Germination resumption under freshwater conditions might have adaptive consequences, as it prevents emergence under harsh environmental conditions, ensuring a higher chance for proper seedling establishment [117,118]. This species can tolerate up to 150 mM NaCl salt stress (at least for two months, Figures 3a and 5b), taking advantage of a prolonged chilling or recovery period, even if in both cases hazard rate for seed germination in high salinity remains low and about 25% and 50% of control values (Table 4). Low salt-stress exposure (10 mM NaCl)—a process known as salt acclimation [119]—improved germination particularly during recovery (Figure 3c). In *Arabidopsis thaliana*, exposure to these same NaCl concentration was found to trigger several physiological responses such as reactive oxygen species (ROS) increase, ROS-scavenging-enzyme activation, compatible solutes accumulation, and Na^+, Cl^-, and K^+ ion-content changes [120,121]. Germination kinetics during recovery from saline stress was similar among NaCl concentrations, and germination probability decrease with salinity was not significant in none of the applied statistical approaches, i.e., survival analysis, critical value determination by hierarchical Bayesian, and regression between final $\hat{F}$ values vs. salinity. Moreover, the similar behavior among salt concentrations during recovery suggested that NaCl had a mainly osmotic effect (at least up to 150 mM and 2-month exposure). This was also confirmed by the observation that 4-week chilled seeds reached (at the end of recovery) similar final values to C_{24} for germination probability ($\hat{F}$) and hazard rate ($\hat{H}$) under all tested NaCl conditions. Thus, moderate–high salinity limited emergence, retaining seed viability. Seed germination resumed under favorable freshwater conditions, highlighting an escape mechanism [122], that might be positive to ensure seedling survival soon after emergence [112], and thus widening regeneration opportunities.

5. Conclusions

F. angustifolia moderate tolerance to salt spray and soil salinity at seedling and plant stage [9–11] could also be confirmed in germination/emergence stage, with absence of germination observed in the field at 60 mM NaCl and estimated at 70–84 mM NaCl depending on interpolation model. Even if a progressively reduction was observed in seed germination with salinity for *F. angustifolia*, emergence took place up to 50 (70) mM NaCl without a significant differences with respect to control. The potential toxicity of high salt levels, hyperosmotic stress, and related secondary stresses, such as oxidative damage [123,124] could be recovered up to the highest NaCl tested levels (150 mM NaCl) after about two months in freshwater conditions. Hence, from an ecological point of view, this study confirmed the importance for overcoming dormancy and complete germination in sub-optimal saline conditions by a prompt germination after (i) a prolonged cold and humid season or (ii) rainy periods such as to reduce soil salinity below threshold critical values.

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