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Salinity delays rhizoid formation and blocks tuberization in the threatened terrestrial orchid Anacamptis coriophora, and chitosan with yeast extract alleviates the damage in vitro

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Research Article

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Salinity delays rhizoid formation and blocks tuberization in the threatened terrestrial orchid *Anacamptis coriophora*, and chitosan with yeast extract alleviates the damage *in vitro*

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Abstract

Background: Tuberous terrestrial orchids of western Asia are dug from the wild to make salep, and asymbiotic seed culture can supply plants for reintroduction. Many candidate sites are degraded or slightly saline, yet nothing is known about how salinity affects early development of these orchids or whether elicitors can soften the damage. Seeds of *Anacamptis coriophora* were sown on P723 medium containing every combination of three NaCl levels (0, 50, 100 mM), three chitosan doses (0, 20, 40 mg L^{-1}) and three yeast extract doses (0, 2, 3 g L^{-1}).

Results: Salinity delayed every germination stage, and rhizoid formation was the most sensitive step (10.5, 24.6 and 52.4 days at 0, 50 and 100 mM). Germination fell from 72% to 60% and 44%, plantlet fresh weight fell by 72% at 50 mM and by 98% at 100 mM, and no tubers formed at 100 mM. NaCl explained 94 to 96% of the variance in plantlet biomass, whereas yeast extract was the main source of variance in chlorophyll content. At 50 mM, malondialdehyde, catalase and superoxide dismutase rose by 63%, 49% and 29%. Without salt, neither elicitor improved germination or growth. Under 50 mM NaCl, 20 mg L^{-1} chitosan shortened the time to protocorm formation from 46 to 32 days, and 40 mg L^{-1} chitosan with 3 g L^{-1} yeast extract tripled plantlet fresh weight and raised tuber fresh weight 4.7-fold. Of 300 trait pairs, 131 were significantly correlated, but only 55 remained so once the NaCl effect was removed; after this correction catalase activity was negatively related to malondialdehyde (partial $r = -0.70$). Principal component analysis, two-way clustering and a salt tolerance index (53 ± 1.2%) all set the chitosan plus yeast extract combination apart from the other salt treatments.

Conclusions: *A. coriophora* is salt-sensitive from germination onwards, and tuber formation is the most vulnerable trait. Elicitors are not needed for routine propagation, but a combination of 40 mg L^{-1} chitosan and 3 g L^{-1} yeast extract restored much of the growth lost at moderate salinity and is a candidate pre-conditioning treatment for plants destined for reintroduction.

Keywords: Orchidaceae; *Orchis coriophora*; Asymbiotic germination; Conservation; Salinity; Elicitation; Chitosan; Yeast extract; Antioxidant enzymes; Salt tolerance index

Background

The Orchidaceae are one of the two largest families of flowering plants, with about 750 genera and more than 25,000 species [1], and they are among the most threatened plant groups worldwide [2]. All orchids are listed by CITES, yet trade and habitat loss continue to reduce many populations [3]. In western Asia and the eastern Mediterranean, tuberous terrestrial orchids face an additional, specific pressure. Their tubers are dug up, boiled, dried and milled to produce salep, a mucilaginous flour used in hot drinks, traditional ice cream and folk medicine, and its value rests on water-soluble polysaccharides whose functional properties, including their fermentation by the gut microbiota, depend strongly on molecular weight [4, 5]. Each tuber represents a whole plant, and thousands of tubers are needed for one kilogram of salep. Field surveys in the Zagros Mountains of Iran have documented intensive and largely unregulated harvesting of many orchid species for this trade [6]. Our earlier work on Iranian salep orchids showed that wild populations differ considerably in seed micromorphology, floral and tuber traits and genetic make-up, and that several populations are small and isolated [7–10]. Comparable genetic structure has been reported for salep orchids in Türkiye [11]. Much of this variation may disappear before it has even been described.

Anacamptis coriophora (L.) R.M. Bateman, Pridgeon & M.W. Chase, long known as *Orchis coriophora* L., is a tuberous terrestrial orchid of damp meadows distributed from western Europe to Iran [12]. It is one of the species collected for salep in western Iran, and its populations are declining in many parts of its range [6, 9]. Like other orchids, it produces minute seeds with an undifferentiated embryo and almost no reserves. In nature these seeds germinate only after infection by a compatible mycorrhizal fungus that supplies carbon and nutrients during the protocorm stage [13, 14]. Asymbiotic in vitro culture, in which a defined medium replaces the fungus, has a long history and is now the most widely used way to produce orchid plants for conservation and reintroduction [15, 16]. Protocols for tuberous terrestrial orchids

65 are still few, however, and germination, protocorm development and tuber formation often
66 remain slow and variable [17, 18]. We have recently developed asymbiotic or large-scale in
67 vitro protocols for *Orchis simia*, *Dactylorhiza umbrosa* and *Himantoglossum affine* [19–21],
68 and in vitro culture has also served the conservation of other threatened medicinal plants of the
69 Zagros region [22, 23]. For *A. coriophora*, the only detailed in vitro study we are aware of
70 focused on basal media and plant growth regulators [24].

71 Salinity is a further concern for this species. Salt-affected soils already cover a large part
72 of the world's land area, and primary salinization is projected to expand during this century in
73 arid and semi-arid regions, including much of western Asia [25]. Meadows and field margins
74 in the Zagros valleys are increasingly exposed to secondary salinization, and rehabilitating such
75 land needs plant material that can establish under saline conditions. Salinity impairs plants first
76 through an osmotic effect and later through ion toxicity and oxidative damage [26, 27]. These
77 effects have been described in detail in crops and in fruit species grown in vitro, where salt
78 reduced growth, chlorophyll content and membrane stability and induced antioxidant enzymes
79 and stress-related genes [28–31]. Terrestrial orchids, by comparison, have received almost no
80 attention. An early report showed that NaCl reduces orchid seed germination in vitro [32], and
81 salt stress has been examined in a few epiphytic orchids [33]. Knowledge of the salt tolerance
82 of *A. coriophora* seedlings is therefore relevant both to the selection of reintroduction sites and
83 to any attempt to cultivate the species outside its natural habitat.

84 Elicitors are compounds that activate defense and stress signaling and, at suitable doses,
85 can also stimulate growth and stress tolerance [34]. We have seen this in our own work. Sodium
86 nitroprusside improved fruit quality and chilling tolerance of tomato [35], while salicylic acid
87 and simvastatin changed regeneration and growth of savory in vitro [23]. Under salinity or
88 drought, potassium silicate, silicon nanoparticles, methyl jasmonate, GABA and biochar
89 strengthened the antioxidant defense of strawberry and ryegrass [29, 31, 36, 37]. Chitosan, the

deacetylated derivative of chitin, is the elicitor most often tested in orchid tissue culture. It has promoted protocorm growth, plantlet development and flowering in several epiphytic orchids [38, 39] and has recently been tested in a Mediterranean terrestrial orchid [40]. In crops, chitosan reduces the effects of salinity by strengthening antioxidant defense, supporting osmotic adjustment and limiting Na^+ accumulation [41, 42]. Yeast extract has two roles. It is a rich source of organic nitrogen and vitamins that is widely added to orchid media [43], and it is also a well-known biotic elicitor [44]. It has also been used to alleviate drought stress in wheat [45]. To our knowledge, neither elicitor has been tested, alone or in combination, on the germination and development of an orchid under salt stress.

The aims of this study were therefore (i) to describe the effects of moderate and severe salinity on the germination stages, protocorm development, plantlet growth, tuber formation and stress physiology of *A. coriophora* in vitro; (ii) to test whether chitosan and yeast extract, alone or combined, can mitigate these effects; (iii) to identify, with effect-size, correlation and multivariate analyses, which traits carry the salt response and which are shaped by the elicitors; and (iv) to identify treatments that could be used to produce plantlets for conservation and reintroduction. Tuber quality and leaf metabolite profiles of the same plantlets will be reported separately.

Results

Course of asymbiotic germination

Seeds passed through the six developmental stages shown in Fig. 1, from the intact seed (stage I) to the emergence of the first leaf from the protocorm (stage VI). All viable seeds began to swell. Stage II was reached after 2 to 4 days at 0 and 50 mM NaCl and after 7 days at 100 mM, with no variation among replicates. For stages III to VI and for germination percentage, all main effects and interactions were significant ($P < 0.01$; Supplementary material: Table S1).

Salinity delayed every stage, and the delay grew as development proceeded (Fig. 2a). Averaged over the elicitor treatments (mean ± SE), stage III was reached after 6.1 ± 0.2, 14.0 ± 1.6 and 24.7 ± 1.0 days at 0, 50 and 100 mM NaCl, stage IV after 10.5 ± 0.1, 24.6 ± 2.1 and 52.4 ± 2.3 days, stage V after 37.0 ± 1.8, 42.5 ± 2.1 and 63.2 ± 0.9 days, and stage VI after 66.1 ± 0.9, 70.5 ± 1.2 and 73.4 ± 1.2 days. Rhizoid formation was therefore the most sensitive step and took five times longer at 100 mM than in the control. The phase from rhizoids to protocorm, on the other hand, became shorter as salinity increased (26.5, 17.9 and 10.8 days), so that the delay accumulated before stage IV was partly made up later.

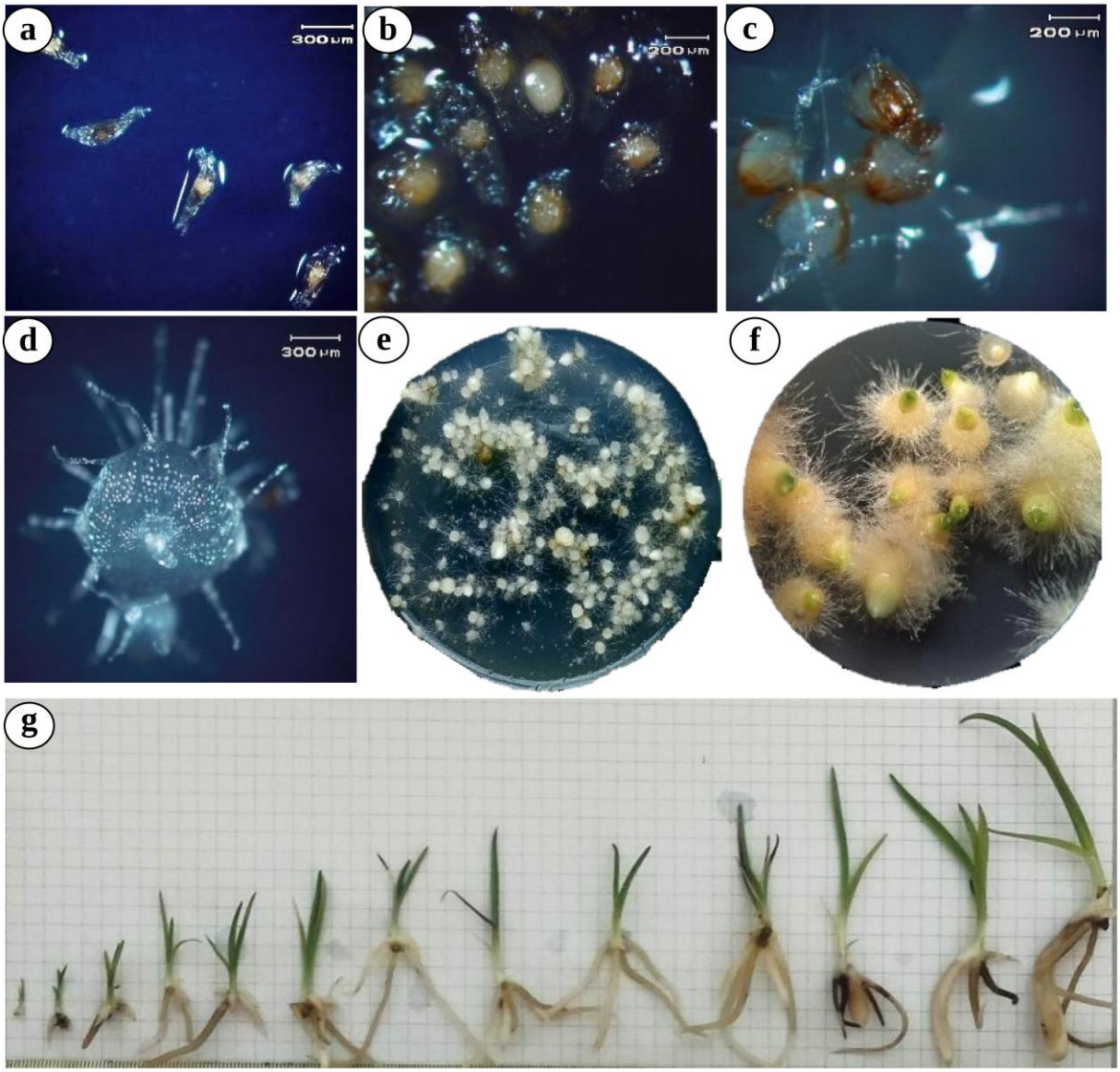


Fig. 1. Stages of asymbiotic seed germination and protocorm development of *Anacamptis coriophora* on P723 medium. (**a**) Stage I, seed with an intact, undifferentiated embryo; (**b**) stage

II, swollen embryo; (**c**) stage III, rupture of the testa; (**d**) stage IV, formation of rhizoids; (**e**) stage V, well-formed protocorm; (**f**) stage VI, emergence of the first shoot. (**g**) The whole process of plantlet development under *in vitro* condition from seed germination to seedling growth.

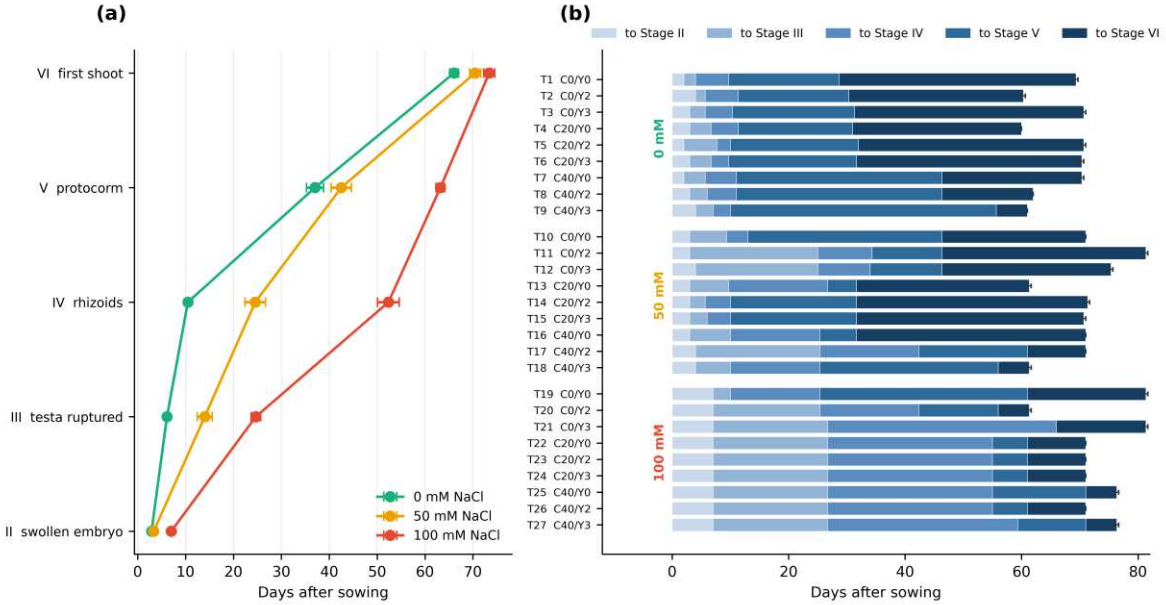


Fig. 2. Time course of asymbiotic germination of *A. coriophora* under NaCl, chitosan and yeast extract. (**a**) Days to reach stages II to VI at 0, 50 and 100 mM NaCl, averaged over the nine chitosan × yeast extract combinations (means ± SE, n = 27). (**b**) Days to each stage in the 27 treatments; segments show the time between successive stages and the error bar the SE of days to stage VI (n = 3). C, chitosan (mg L^{-1}); Y, yeast extract (g L^{-1}).

The effect of the elicitors depended on salinity (Table 1; Fig. 2b; Fig. 3). Without NaCl, the untreated control (T1) reached stage V fastest (28.7 ± 0.3 days), and 40 mg L^{-1} chitosan combined with yeast extract (T8 and T9) was slowest (46.3 to 55.7 days). At 50 mM NaCl, the pattern reversed. The three treatments with 20 mg L^{-1} chitosan (T13 to T15) and 40 mg L^{-1} chitosan alone (T16) reached stage V after 31.7 days, compared with 46.3 ± 0.3 days in the stressed control (T10), and T14 and T15 formed rhizoids after only 10 days, as fast as the unstressed control. Yeast extract without chitosan (T11 and T12) delayed stage III to 25 days at this salinity.

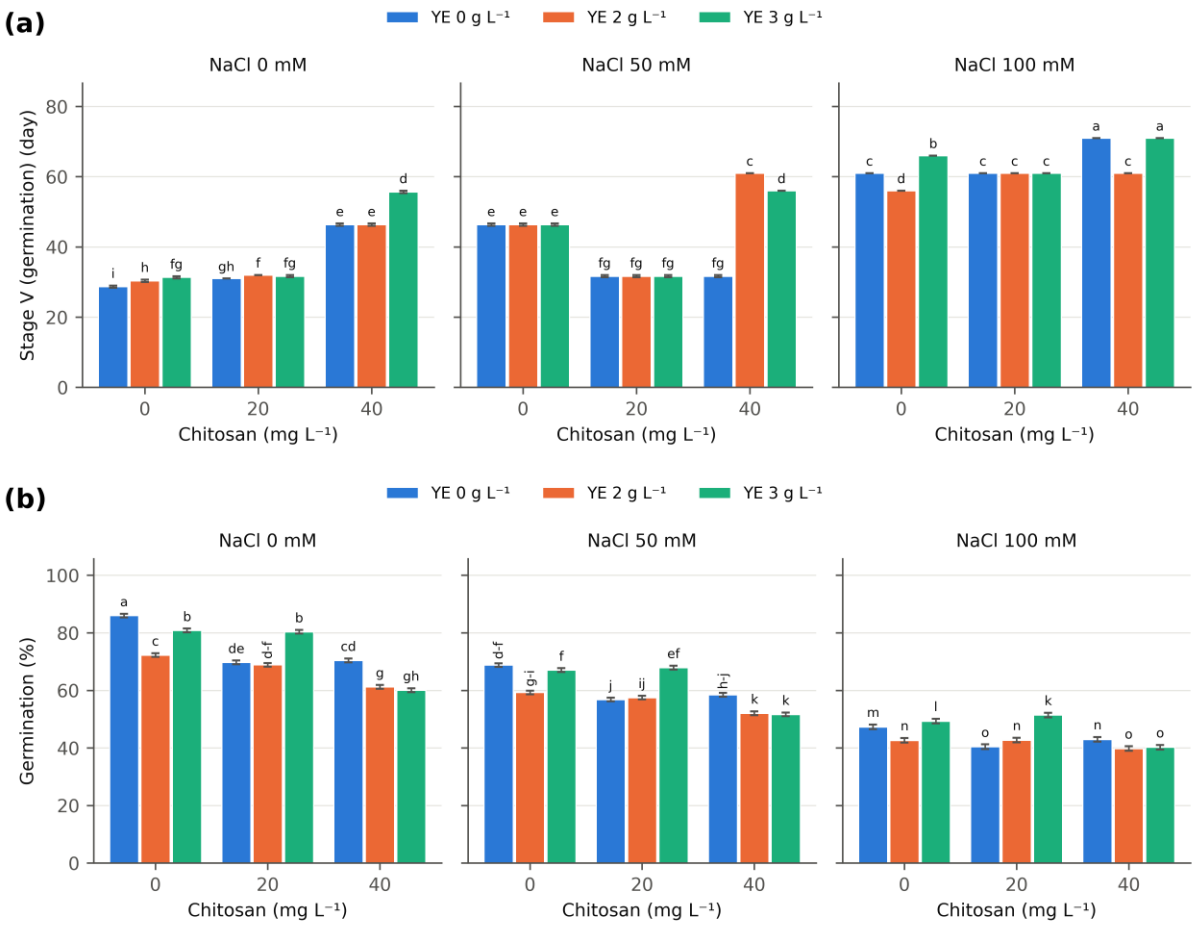


Fig. 3. Days to protocorm formation and final germination of *A. coriophora*. (**a**) Days to stage V and (**b**) final germination percentage under NaCl, chitosan and yeast extract (YE). Bars are means ± SE (n = 3 for **a**, n = 5 for **b**); bars sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test).

Germination fell from 72.2 ± 1.3% at 0 mM to 59.9 ± 1.0% at 50 mM and 44.1 ± 0.6% at 100 mM NaCl. The highest value of the experiment (86.0 ± 0.6%) was obtained in the unstressed control, and no elicitor treatment exceeded it. At 0 and 50 mM, 40 mg L^{-1} chitosan combined with yeast extract gave the lowest germination (60.1 to 61.2% and about 52%). Only at 100 mM did the elicitors raise germination above the stressed control (47.3 ± 0.8%), with 51.4 ± 0.8% for 20 mg L^{-1} chitosan plus 3 g L^{-1} yeast extract (T24) and 49.3 ± 0.8% for 3 g L^{-1} yeast extract alone (T21).

Table 1. Days to protocorm formation (stage V), final germination and plantlet growth of *Anacamptis coriophora* under 27 combinations of NaCl, chitosan and yeast extract (YE)

Trt	NaCl (mM)	Chitosan (mg L^{-1})	YE (g L^{-1})	Days to stage V (day)	Germination (%)	Plantlet height (mm)	Plantlet FW (g)	Plantlet DW (g)
T1	0	0	0	28.7 ± 0.3 i	86.0 ± 0.6 a	182 ± 1 a	2.19 ± 0.08 a	0.243 ± 0.014 a
T2	0	0	2	30.3 ± 0.3 h	72.3 ± 0.6 c	137 ± 1 f	1.79 ± 0.09 c	0.204 ± 0.011 b
T3	0	0	3	31.3 ± 0.3 fg	80.8 ± 0.6 b	155 ± 1 e	2.19 ± 0.11 a	0.185 ± 0.012 b
T4	0	20	0	31.0 ± 0.0 gh	69.7 ± 0.6 de	131 ± 1 g	1.21 ± 0.05 d	0.118 ± 0.007 d
T5	0	20	2	32.0 ± 0.0 f	68.9 ± 0.6 d-f	166 ± 1 c	1.84 ± 0.07 bc	0.180 ± 0.010 bc
T6	0	20	3	31.7 ± 0.3 fg	80.4 ± 0.6 b	138 ± 1 f	1.36 ± 0.07 d	0.155 ± 0.007 c
T7	0	40	0	46.3 ± 0.3 e	70.4 ± 0.6 cd	132 ± 1 g	0.947 ± 0.033 e	0.110 ± 0.006 d
T8	0	40	2	46.3 ± 0.3 e	61.2 ± 0.6 g	179 ± 1 b	1.97 ± 0.08 a-c	0.191 ± 0.011 b
T9	0	40	3	55.7 ± 0.3 d	60.1 ± 0.6 gh	158 ± 1 d	2.07 ± 0.11 ab	0.209 ± 0.012 ab
T10	50	0	0	46.3 ± 0.3 e	68.8 ± 0.6 d-f	78.3 ± 0.5 k	0.332 ± 0.015 i	0.045 ± 0.002 g
T11	50	0	2	46.3 ± 0.3 e	59.3 ± 0.6 g-i	94.1 ± 0.5 i	0.420 ± 0.015 h	0.050 ± 0.003 g
T12	50	0	3	46.3 ± 0.3 e	67.1 ± 0.6 f	78.7 ± 0.5 k	0.532 ± 0.026 g	0.061 ± 0.003 f
T13	50	20	0	31.7 ± 0.3 fg	56.8 ± 0.6 j	89.7 ± 0.5 j	0.469 ± 0.024 h	0.059 ± 0.003 f
T14	50	20	2	31.7 ± 0.3 fg	57.5 ± 0.6 ij	80.3 ± 0.6 k	0.328 ± 0.012 i	0.031 ± 0.001 h
T15	50	20	3	31.7 ± 0.3 fg	67.9 ± 0.6 ef	88.2 ± 0.6 j	0.615 ± 0.031 f	0.078 ± 0.004 e
T16	50	40	0	31.7 ± 0.3 fg	58.5 ± 0.6 h-j	73.9 ± 0.4 l	0.446 ± 0.016 h	0.059 ± 0.003 f
T17	50	40	2	61.0 ± 0.0 c	52.0 ± 0.6 k	58.2 ± 0.4 m	0.236 ± 0.009 j	0.033 ± 0.002 h
T18	50	40	3	56.0 ± 0.0 d	51.6 ± 0.6 k	121 ± 1 h	1.00 ± 0.04 e	0.123 ± 0.007 d
T19	100	0	0	61.0 ± 0.0 c	47.3 ± 0.8 m	17.7 ± 0.1 o	0.023 ± 0.001 m	0.003 ± 0.000 m
T20	100	0	2	56.0 ± 0.0 d	42.6 ± 0.8 n	14.7 ± 0.1 p	0.039 ± 0.001 k	0.006 ± 0.000 i
T21	100	0	3	66.0 ± 0.0 b	49.3 ± 0.8 l	13.8 ± 0.1 p	0.030 ± 0.001 l	0.005 ± 0.000 j
T22	100	20	0	61.0 ± 0.0 c	40.4 ± 0.8 o	14.1 ± 0.1 p	0.029 ± 0.001 l	0.006 ± 0.000 i
T23	100	20	2	61.0 ± 0.0 c	42.7 ± 0.8 n	20.2 ± 0.1 n	0.030 ± 0.001 l	0.004 ± 0.000 k
T24	100	20	3	61.0 ± 0.0 c	51.4 ± 0.8 k	12.7 ± 0.1 p	0.025 ± 0.001 m	0.003 ± 0.000 lm
T25	100	40	0	71.0 ± 0.0 a	43.0 ± 0.8 n	13.3 ± 0.1 p	0.016 ± 0.001 n	0.004 ± 0.000 kl
T26	100	40	2	61.0 ± 0.0 c	39.8 ± 0.8 o	14.5 ± 0.1 p	0.030 ± 0.001 l	0.004 ± 0.000 jk
T27	100	40	3	71.0 ± 0.0 a	40.2 ± 0.8 o	9.06 ± 0.07 q	0.015 ± 0.001 n	0.002 ± 0.000 n

Values are means ± standard error (SE). Within a column, means sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test; letter ranges such as a-c include all intermediate letters). n = 3 for days to stage V and n = 5 for the other traits. Plantlet fresh (FW) and dry weight (DW) were analyzed after log10 transformation; untransformed means and SE are shown.

Protocorm and shoot development

The three-way interaction was significant for all protocorm and shoot traits (Supplementary material: Table S1). Unlike the other growth traits, protocorm size did not decrease under salinity: protocorm length rose from 3.57 ± 0.08 mm at 0 mM to 4.04 ± 0.05 mm at 100 mM. The largest protocorms were formed in T18 (50 mM NaCl with 40 mg L^{-1} chitosan and 3 g L^{-1} yeast extract; 25.8 ± 1.6 mg fresh weight). The appearance of plantlets in representative treatments is shown in Fig. 4.

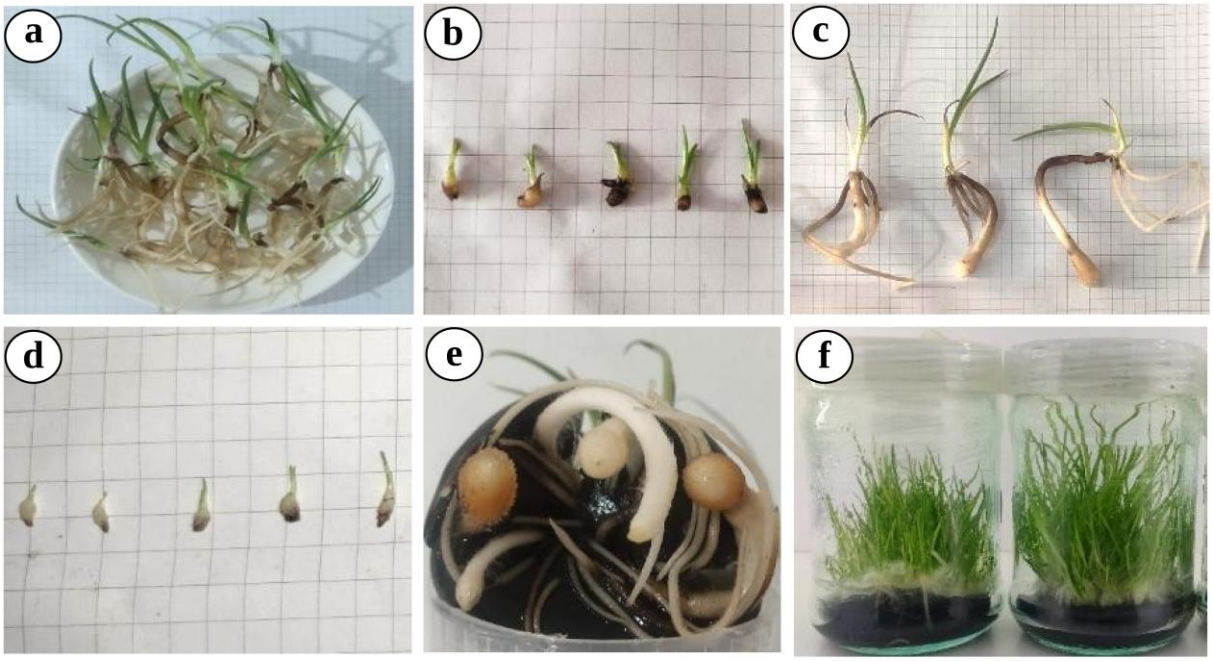


Fig. 4. Plantlet development of *A. coriophora* under salinity and elicitors. (**a**) Unstressed control (T1) with roots and tuber; (**b**) stressed control at 50 mM NaCl (T10); (**c**) 50 mM NaCl with 40 mg L^{-1} chitosan and 3 g L^{-1} yeast extract (T18); (**d**) stressed control at 100 mM NaCl (T19); (**e**) tubers formed in T1, T10 and T18; (**f**) culture vessels at harvest.

Plantlet height averaged 153 ± 3, 84.8 ± 2.5 and 14.4 ± 0.4 mm at 0, 50 and 100 mM NaCl, which corresponds to reductions of 45% and 91% (Fig. 5a). Stem diameter fell from 3.63 to 2.72 and 1.40 mm, while leaf number was slightly higher at 50 mM (3.62 ± 0.07) than at 0 mM (3.18 ± 0.09), so that moderately stressed plantlets formed more but smaller leaves. Without NaCl, the tallest plantlets were found in the control (182 ± 1 mm) and in T8 (40 mg L^{-1} chitosan with 2 g L^{-1} yeast extract; 179 ± 1 mm), and chitosan alone gave the shortest plantlets (T4 and T7, 131 to 132 mm). Under 50 mM NaCl, T18 was the exception, with plantlets 121 ± 1 mm tall, 55% taller than the stressed control (78.3 ± 0.5 mm), together with the longest shoots, the thickest stems and the largest leaves at this salinity level. The closely related T17, which differed only by having 2 g L^{-1} instead of 3 g L^{-1} yeast extract, was the poorest treatment at 50 mM (58.2 ± 0.4 mm).

Roots and tubers

At 0 and 50 mM NaCl, the three-way interaction was significant for all root and tuber traits (Supplementary material: Table S2). Salinity did not change root number (3.62 and 3.58 roots per plantlet) but reduced root length from 60.8 ± 1.7 to 26.5 ± 1.0 mm and root dry weight by 46% (Table 2). At 50 mM, T18 formed the longest roots (40.0 ± 1.5 mm, compared with 27.1 ± 1.3 mm in the stressed control) and T17 the shortest (13.0 ± 0.5 mm) (Fig. 6a). At 100 mM NaCl, short roots (1.8 to 4.4 mm) formed without chitosan or with 20 mg L^{-1} chitosan, and no roots formed with 40 mg L^{-1} chitosan (Supplementary material: Table S4).

Table 2. Root, tuber and leaf physiological traits of *A. coriophora* plantlets at 0 and 50 mM NaCl combined with chitosan and yeast extract (YE)

Trt	NaCl (mM)	Chitosan (mg L^{-1})	YE (g L^{-1})	Root length (mm)	Tuber length (mm)	Tuber FW (g)	Tuber DW (g)	Total chlorophyll (mg g^{-1} FW)	Proline (µg g^{-1} FW)	Leaf soluble carbohydrate (µg g^{-1} FW)
T1	0	0	0	74.1 ± 3.3 a	86.1 ± 3.4 a	1.24 ± 0.06 ab	0.113 ± 0.007 b	1.48 ± 0.04 k	43.0 ± 1.1 g	323 ± 11 f
T2	0	0	2	53.7 ± 2.5 ef	57.6 ± 2.7 c	1.09 ± 0.05 bc	0.126 ± 0.008 a	3.49 ± 0.10 fg	88.4 ± 2.4 b	532 ± 16 cd
T3	0	0	3	72.8 ± 2.7 a	68.6 ± 3.7 b	1.32 ± 0.09 a	0.106 ± 0.007 b	4.28 ± 0.05 e	34.0 ± 0.8 i	783 ± 28 a
T4	0	20	0	56.9 ± 2.2 de	47.6 ± 2.2 d	0.662 ± 0.032 e	0.061 ± 0.003 d	4.31 ± 0.10 e	62.3 ± 1.4 e	533 ± 14 cd
T5	0	20	2	61.4 ± 2.6 cd	87.3 ± 3.4 a	1.05 ± 0.06 c	0.089 ± 0.005 c	6.62 ± 0.12 b	61.9 ± 1.4 e	683 ± 22 b
T6	0	20	3	45.6 ± 2.3 gh	49.0 ± 2.6 d	0.791 ± 0.034 d	0.085 ± 0.004 c	4.94 ± 0.06 d	73.7 ± 2.3 cd	499 ± 18 d
T7	0	40	0	49.0 ± 2.3 fg	36.5 ± 1.9 e	0.486 ± 0.021 f	0.056 ± 0.003 de	1.77 ± 0.01 j	37.4 ± 0.8 hi	790 ± 18 a
T8	0	40	2	64.2 ± 3.4 bc	71.9 ± 3.5 b	1.18 ± 0.06 a-c	0.107 ± 0.005 b	4.50 ± 0.08 e	36.7 ± 0.9 hi	556 ± 20 c
T9	0	40	3	69.9 ± 3.3 ab	70.8 ± 2.9 b	1.25 ± 0.07 ab	0.105 ± 0.006 b	5.90 ± 0.13 c	62.5 ± 1.3 e	447 ± 11 e
T10	50	0	0	27.1 ± 1.3 i	17.5 ± 0.9 ij	0.088 ± 0.005 k	0.011 ± 0.001 h	2.23 ± 0.02 i	55.9 ± 1.6 f	220 ± 7 gh
T11	50	0	2	29.4 ± 1.2 i	21.8 ± 1.0 g-i	0.146 ± 0.006 j	0.017 ± 0.001 h	3.53 ± 0.11 fg	111 ± 3 a	332 ± 11 f
T12	50	0	3	24.1 ± 0.9 i	25.1 ± 1.0 f-h	0.162 ± 0.009 j	0.013 ± 0.001 h	5.07 ± 0.04 d	41.7 ± 1.1 gh	249 ± 7 g
T13	50	20	0	28.4 ± 1.1 i	29.5 ± 1.5 f	0.345 ± 0.019 h	0.035 ± 0.002 fg	3.61 ± 0.10 f	78.7 ± 1.6 c	127 ± 3 i
T14	50	20	2	25.5 ± 1.4 i	12.2 ± 0.5 jk	0.062 ± 0.004 l	0.005 ± 0.000 h	2.30 ± 0.03 i	75.1 ± 1.9 cd	187 ± 6 h
T15	50	20	3	26.6 ± 0.9 i	21.1 ± 0.9 hi	0.250 ± 0.011 i	0.030 ± 0.002 g	2.89 ± 0.09 h	87.5 ± 2.7 b	144 ± 3 i
T16	50	40	0	24.2 ± 1.0 i	28.5 ± 1.5 fg	0.243 ± 0.012 i	0.032 ± 0.002 g	1.83 ± 0.01 j	45.8 ± 1.1 g	78.8 ± 2.3 j
T17	50	40	2	13.0 ± 0.5 j	7.50 ± 0.38 k	0.057 ± 0.003 l	0.006 ± 0.000 h	3.27 ± 0.03 g	43.1 ± 1.3 g	201 ± 6 h
T18	50	40	3	40.0 ± 1.5 h	40.2 ± 1.9 e	0.415 ± 0.019 g	0.045 ± 0.002 ef	7.17 ± 0.19 a	71.9 ± 1.6 d	305 ± 10 f

Values are means ± standard error (SE). Within a column, means sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test; letter ranges such as a-c include all intermediate letters). n = 5 for root and tuber traits and n = 3 for leaf traits. Tuber FW was analyzed after log10 transformation. No tubers formed at 100 mM NaCl.

Every plantlet that formed a tuber at 0 and 50 mM NaCl formed exactly one, and no tuber formed at 100 mM NaCl in any treatment. At 50 mM, tuber length fell by 65%, tuber fresh weight by 81% and tuber dry weight by 77%. Without salt, the largest tubers were formed by the control and by 3 g L^{-1} yeast extract alone (1.24 ± 0.06 and 1.32 ± 0.09 g fresh weight), and the smallest by 40 mg L^{-1} chitosan alone. Under 50 mM NaCl, T18 formed tubers with a fresh weight of 0.415 ± 0.019 g and a dry weight of 44.6 ± 2.1 mg, which is 4.7 and 3.9 times the

values of the stressed control (0.088 ± 0.005 g and 11.3 ± 0.7 mg) (Fig. 6b). Chitosan alone also improved tuber dry weight under salinity, to 34.9 ± 1.6 mg at 20 mg L^{-1} (T13) and 31.5 ± 1.9 mg at 40 mg L^{-1} (T16), about three times the stressed control, whereas the combinations of chitosan with 2 g L^{-1} yeast extract (T14 and T17) produced the smallest tubers (4.8 and 6.2 mg dry weight).

Plantlet biomass

Plantlet fresh weight fell from 1.73 ± 0.07 g at 0 mM to 0.487 ± 0.033 g at 50 mM and 0.026 ± 0.001 g at 100 mM NaCl, and plantlet dry weight from 177 ± 7 to 60 ± 4 and 4 mg, so that plantlets at 100 mM reached only about 2% of the control biomass (Table 1; Fig. 5b). Without salt, the heaviest plantlets were produced by the control and by 3 g L^{-1} yeast extract alone (2.19 ± 0.08 and 2.19 ± 0.11 g), while chitosan alone reduced biomass (1.21 ± 0.05 g in T4 and 0.95 ± 0.03 g in T7). At 50 mM NaCl, T18 reached a fresh weight of 1.00 ± 0.04 g, three times that of the stressed control (0.332 ± 0.015 g), and a dry weight of 123 ± 7 mg compared with 45.2 ± 2.2 mg. It thus reached nearly half of the unstressed control fresh weight, whereas the stressed control reached only 15%. At 100 mM, the best biomass was obtained with 2 g L^{-1} yeast extract alone (T20; 38.7 ± 1.3 mg fresh weight compared with 23.2 ± 0.9 mg in T19).

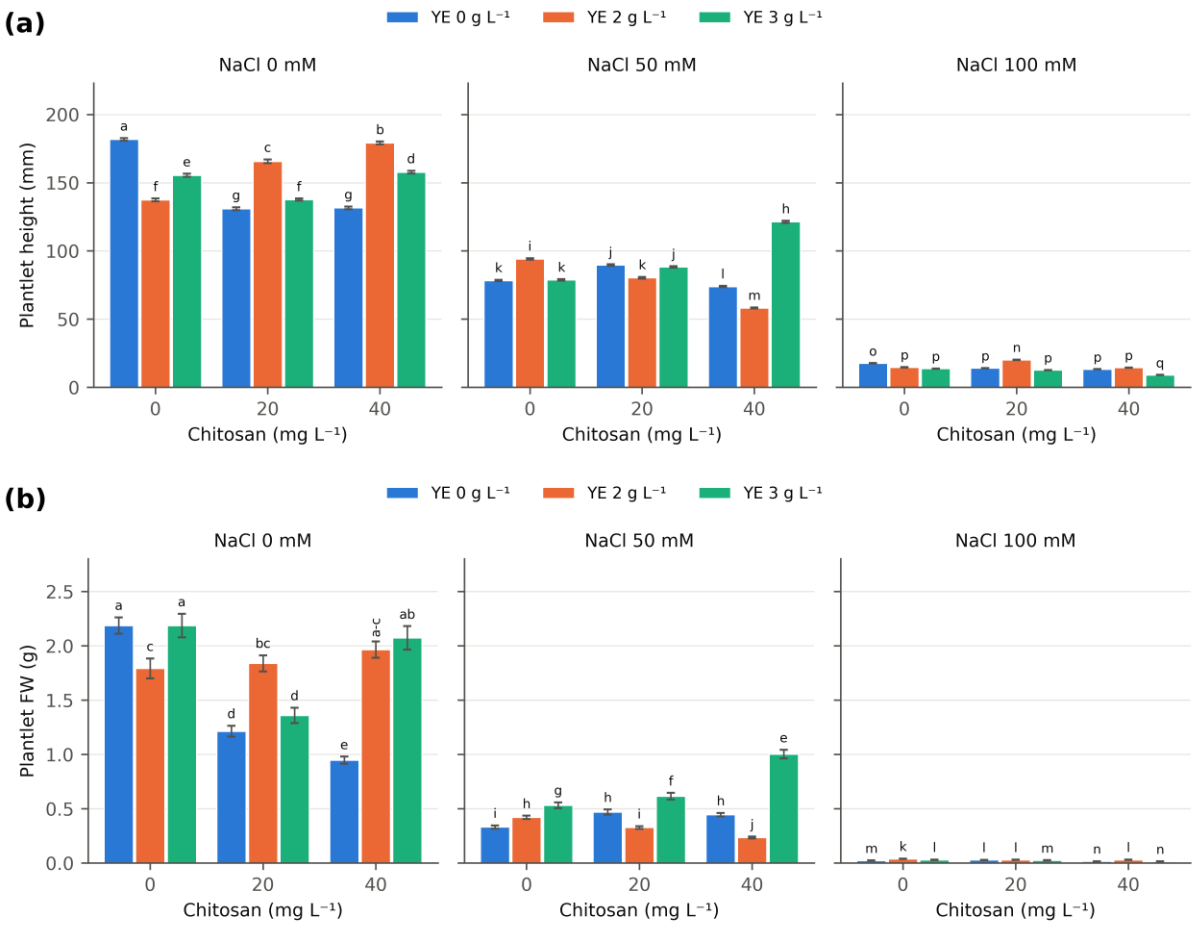


Fig. 5. Plantlet height and fresh weight of *A. coriophora*. (**a**) Plantlet height and (**b**) plantlet fresh weight (FW) under NaCl, chitosan and yeast extract (YE). Bars are means ± SE (n = 5); bars sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test). Fresh weight was analysed after $\log_{10}$ transformation.

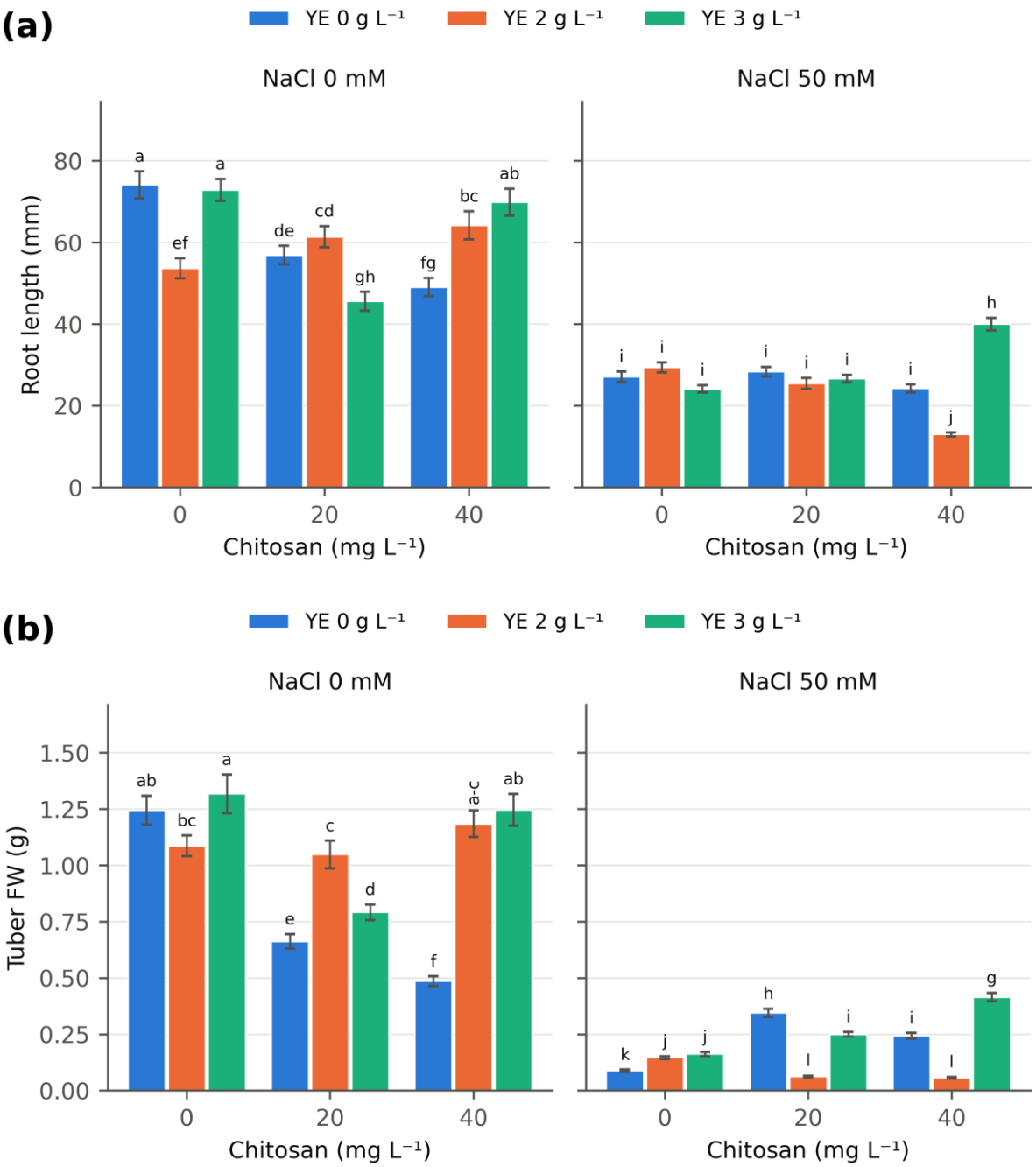


Fig. 6. Root length and tuber fresh weight of *A. coriophora* plantlets at 0 and 50 mM NaCl. (**a**) Mean root length and (**b**) tuber fresh weight with chitosan and yeast extract (YE). No tubers formed at 100 mM NaCl. Bars are means ± SE (n = 5); bars sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test).

Photosynthetic pigments and osmolytes

All effects were significant for chlorophylls and carotenoids (Supplementary material: Table S2). Salinity reduced chlorophyll a by 28%, total chlorophyll by 15% and carotenoids by 40%. Yeast extract had a strong positive effect, raising mean total chlorophyll from 2.54 ± 0.26

mg g^{-1} FW without yeast extract to 3.95 ± 0.33 and 5.04 ± 0.32 mg g^{-1} FW with 2 and 3 g L^{-1}. The untreated control had the lowest total chlorophyll of all treatments (1.48 ± 0.04 mg g^{-1} FW), whereas T5 (20 mg L^{-1} chitosan with 2 g L^{-1} yeast extract) had 6.62 ± 0.12 mg g^{-1} FW without salt, and T18 had the highest value of the experiment (7.17 ± 0.19 mg g^{-1} FW) (Fig. 7a). T18 also had clearly the highest chlorophyll b (5.59 ± 0.21 mg g^{-1} FW) and the lowest carotenoid content (0.053 ± 0.003 mg g^{-1} FW).

Proline increased by 22% under salinity. The highest proline content was found with 2 g L^{-1} yeast extract alone at 50 mM NaCl (T11; 111 ± 3 µg g^{-1} FW), followed by the same yeast extract dose without salt (T2; 88 ± 2 µg g^{-1} FW), while T18 had a moderate value (72 ± 2 µg g^{-1} FW) (Fig. 7b). Leaf soluble carbohydrate fell by 64% at 50 mM NaCl. Without salt it was highest with 40 mg L^{-1} chitosan alone (T7) and 3 g L^{-1} yeast extract alone (T3), more than twice the control, and at 50 mM it was highest in T11 and T18 (Table 2).

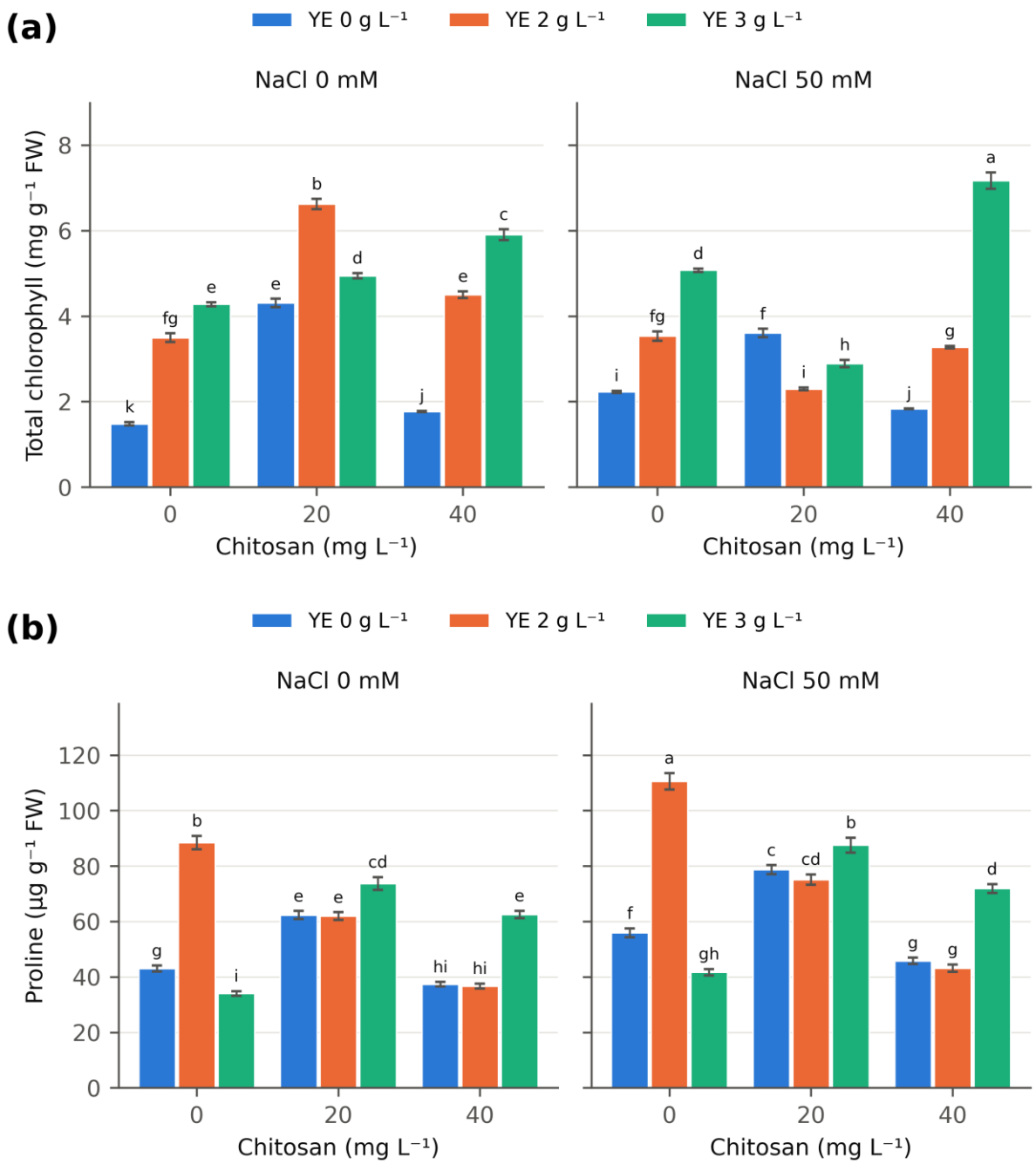


Fig. 7. Total chlorophyll and proline content of *A. coriophora* plantlets at 0 and 50 mM NaCl. (**a**) Total chlorophyll and (**b**) proline with chitosan and yeast extract (YE). Bars are means ± SE (n = 3); bars sharing a letter do not differ at P ≤ 0.05 (Duncan's multiple range test).

Oxidative stress and antioxidant enzymes

MDA, CAT and SOD responded mainly to salinity ($P < 0.001$), and none of the interactions was significant (Supplementary material: Table S2). Among the elicitors, only yeast extract had a significant effect, on SOD ($P = 0.029$), with trends for CAT ($P = 0.081$) and MDA ($P = 0.064$). The results are therefore presented as main effects (Table 3; Fig. 8). Salinity

increased MDA by 63%, CAT activity by 49% and SOD activity by 29%. Yeast extract at 3 g L^{-1} increased SOD significantly relative to no yeast extract (5.84 ± 0.21 compared with 5.26 ± 0.21 U mg^{-1} protein) and tended to increase CAT and lower MDA. At 50 mM NaCl, the lowest MDA was recorded in T18 and the highest in the stressed control (a 24% difference), but this difference was not significant within the factorial model.

Table 3. Main-effect means of NaCl, chitosan and yeast extract on malondialdehyde (MDA) content and catalase (CAT) and superoxide dismutase (SOD) activities of *A. coriophora* plantlets

Factor	Level	MDA	CAT	SOD
NaCl (mM)	0	39.84 ± 1.03 b	5.93 ± 0.121 b	4.89 ± 0.108 b
	50	64.72 ± 1.83 a	8.85 ± 0.186 a	6.29 ± 0.127 a
Chitosan (mg L^{-1})	0	54.57 ± 3.77 a	7.22 ± 0.340 a	5.39 ± 0.212 a
	20	53.02 ± 3.67 a	7.41 ± 0.421 a	5.61 ± 0.224 a
	40	49.26 ± 2.98 a	7.54 ± 0.440 a	5.77 ± 0.225 a
Yeast extract (g L^{-1})	0	55.58 ± 3.92 a	7.02 ± 0.394 b	5.26 ± 0.214 b
	2	51.94 ± 3.50 ab	7.47 ± 0.403 ab	5.67 ± 0.219 ab
	3	49.32 ± 2.95 b	7.68 ± 0.399 a	5.84 ± 0.214 a

Values are means ± SE (n = 27 for NaCl levels and n = 18 for chitosan and yeast extract levels). Within each factor and column, means sharing a letter do not differ at $P \leq 0.05$ (Duncan's multiple range test). None of the interactions was significant (Supplementary material: Table S2). MDA, malondialdehyde (nmol g^{-1} FW); CAT, catalase (U mg^{-1} protein); SOD, superoxide dismutase (U mg^{-1} protein).

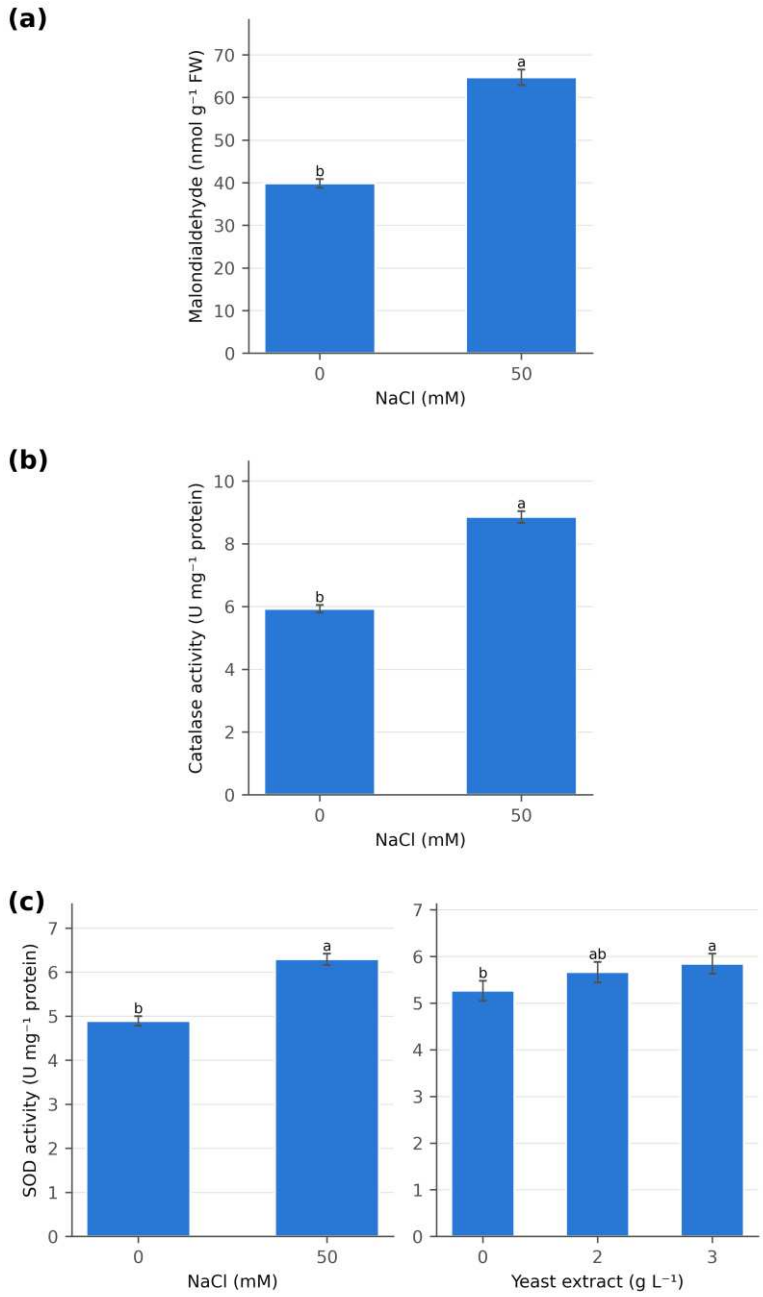


Fig. 8. Main effects of NaCl, chitosan and yeast extract on oxidative stress markers of *A. coriophora* plantlets. (**a**) Malondialdehyde (MDA) content, (**b**) catalase (CAT) activity and (**c**) superoxide dismutase (SOD) activity. Bars are means ± SE; bars sharing a letter within a panel do not differ at $P \leq 0.05$ (Duncan's multiple range test).

Partitioning of variance among the factors

The share of the total sum of squares attributable to each source showed which traits were governed by salinity and which by the elicitors (Fig. 9; Supplementary material: Table S8). NaCl alone explained 94 to 96% of the variation in plantlet height, fresh weight and dry weight, 68 to 77% in root length and tuber traits, 63 to 78% in the days to stages III to V and 76% in

germination. Its share was also large for MDA (73%), CAT (77%), leaf soluble carbohydrate (71%) and SOD (58%), although for the three oxidative traits a further 18 to 32% remained unexplained, reflecting the smaller number of replicates. Chitosan contributed most to the days to stage V (17%), germination (11%) and proline (21%). Yeast extract was the largest single source of variation in total chlorophyll (40%), chlorophyll a (28%) and chlorophyll b (22%), where NaCl explained only 1 to 14%. In several traits the interactions dominated: the chitosan × yeast extract interaction accounted for 59% of the variation in proline and 25 to 30% in total chlorophyll and chlorophyll b, the three-way interaction for 41% of leaf protein, and the interactions together for 70% of protocorm fresh weight and 67% of the days to stage VI.

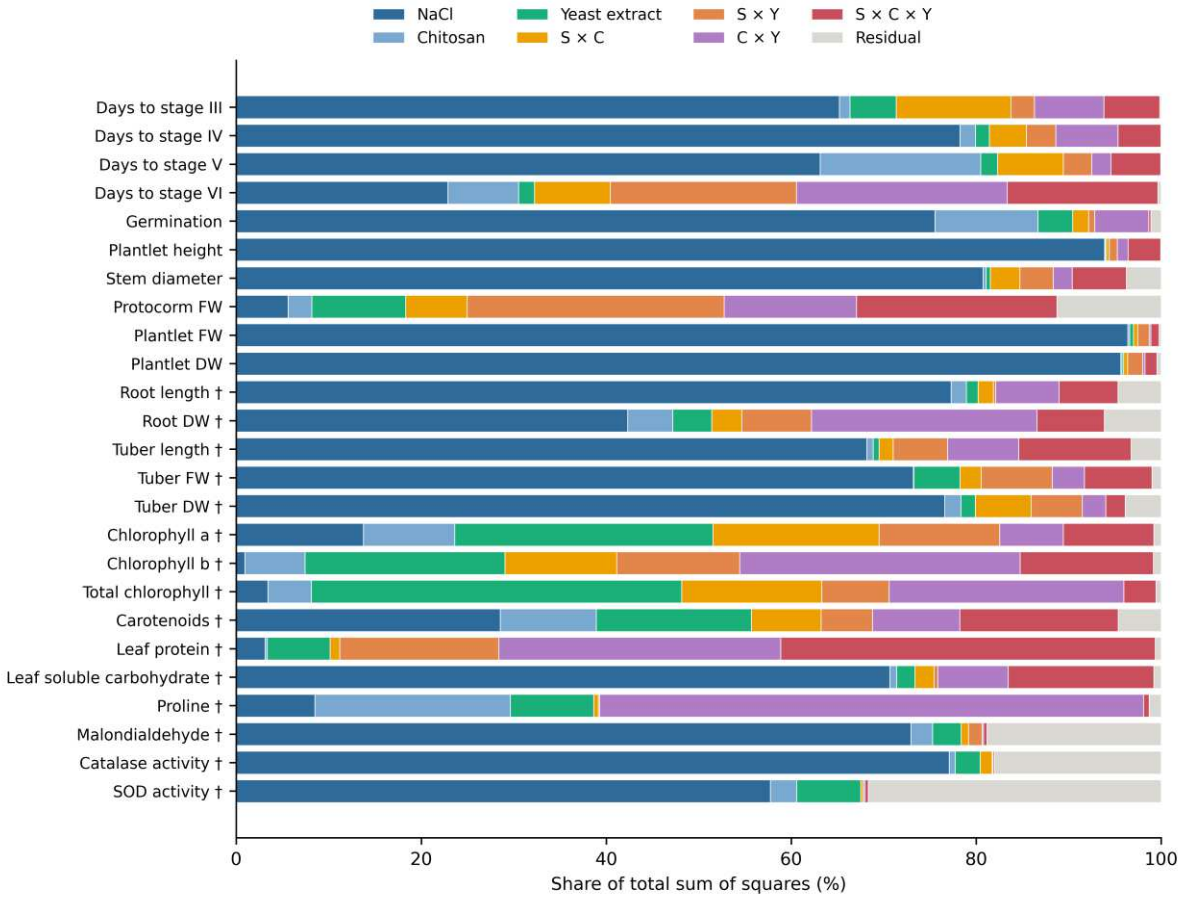


Fig. 9. Partitioning of variance among sources of variation. Share of the total sum of squares (%) explained by NaCl (S), chitosan (C), yeast extract (Y), their interactions and the residual for 25 traits of *A. coriophora*. Traits marked † were analysed in the 2 × 3 × 3 factorial (NaCl 0 and 50 mM); the others in the 3 × 3 × 3 factorial. FW, fresh weight; DW, dry weight.

Correlations among traits

Across the 18 treatments at 0 and 50 mM NaCl, 131 of the 300 pairwise Pearson correlations among 25 traits were significant at $P < 0.05$, and 107 remained so after false discovery rate control (Fig. 10, lower triangle; Supplementary material: Table S9). Growth and tuber traits were closely interrelated (for example, plantlet and tuber fresh weight, $r = 0.99$; root length and tuber fresh weight, $r = 0.96$), and all of them were negatively correlated with MDA, CAT and SOD (for example, plantlet fresh weight and MDA, $r = -0.87$; root length and CAT, $r = -0.87$) and with the days to stages III and IV. CAT and SOD were almost perfectly correlated ($r = 0.98$), and both were positively correlated with MDA ($r = 0.86$ for CAT).

Because these correlations could arise only from the shared response to NaCl, they were recomputed after the salinity effect had been removed (Fig. 10, upper triangle). Only 55 pairs remained significant (36 after false discovery rate control). Correlations within the growth block survived (plantlet and tuber fresh weight, partial $r = 0.96$; root length and tuber fresh weight, partial $r = 0.78$), as did those between CAT and SOD (0.91), between days to stages III and IV (0.87) and between chlorophyll b and protocorm fresh weight (0.71, unchanged). In contrast, the negative relationships of growth traits with MDA, CAT and SOD disappeared (for example, plantlet fresh weight and MDA, partial $r = -0.25$, $P = 0.34$; root length and CAT, partial $r = 0.04$), as did the links of growth with germination percentage and with leaf soluble carbohydrate. The relationship between CAT and MDA reversed sign: within a salinity level, treatments with higher CAT activity had lower MDA (partial $r = -0.70$, $P = 0.002$). Proline was not correlated with plantlet fresh weight either before or after the correction ($r = -0.27$ and -0.02).

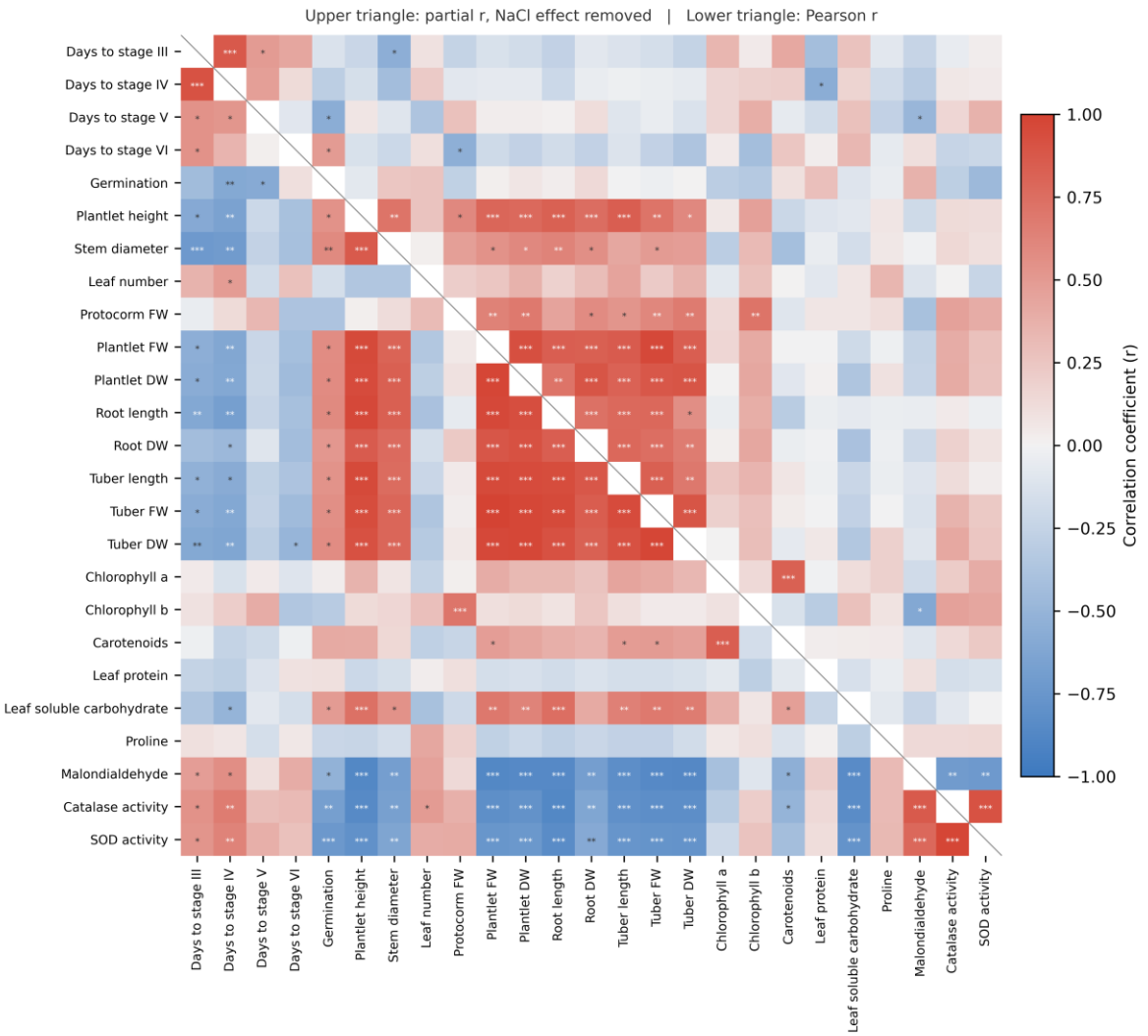


Fig. 10. Correlations among germination, growth and physiological traits of *A. coriophora*. Lower triangle: Pearson correlation coefficients among the means of the 18 treatments at 0 and 50 mM NaCl. Upper triangle: partial correlation coefficients after removal of the NaCl effect (df = 15). *, ** and ***: significant at $P < 0.05$, 0.01 and 0.001 (unadjusted). FW, fresh weight; DW, dry weight.

Two-way clustering and principal component analysis

The two-way clustered heat map grouped both treatments and traits (Fig. 11). Treatments split first by salinity. Within the 50 mM group, T18 formed a separate branch with high values for protocorm fresh weight and chlorophyll b and the least depressed growth; T11, T12 and T17 were grouped by their long delays to stages III and IV; and T10 and T13 to T16 formed a third group. Within the unstressed group, the control (T1) joined T8 and T9, which combined 40 mg L^{-1} chitosan with yeast extract, and the remaining unstressed treatments formed a second group. Traits fell into a growth and tuber cluster that also contained germination percentage and leaf

soluble carbohydrate, a stress cluster containing MDA, CAT, SOD, proline, leaf protein, leaf number and the days to germination stages, a pigment cluster of chlorophyll a and carotenoids, and a pair formed by protocorm fresh weight and chlorophyll b.

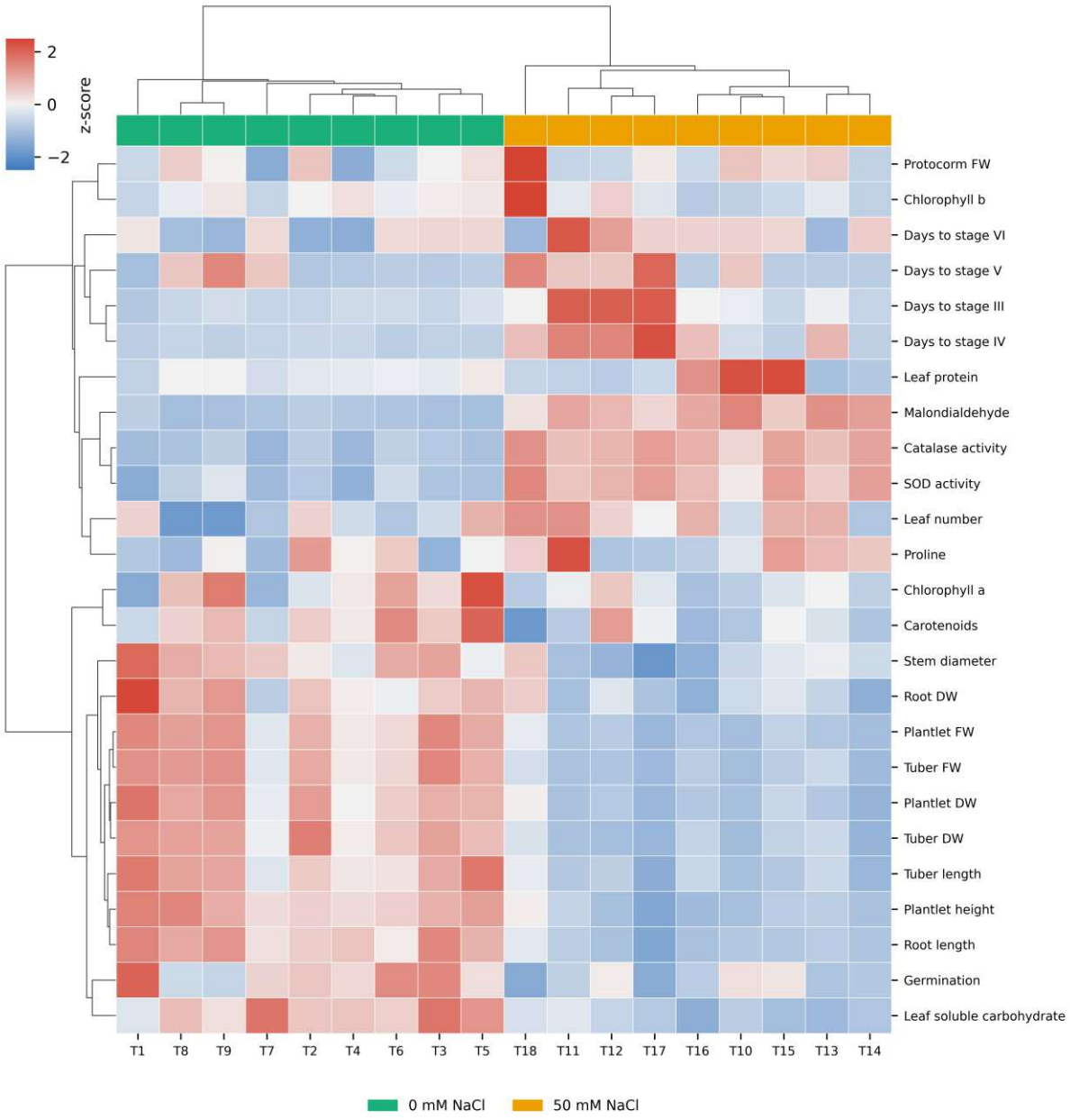


Fig. 11. Two-way hierarchical cluster analysis of treatments and traits. Heat map of z-standardized means of 25 traits in the 18 treatments at 0 and 50 mM NaCl of *A. coriophora*, with treatments (columns) and traits (rows) clustered by Ward's method on Euclidean distances. The colour bar above the heat map indicates the NaCl level. FW, fresh weight; DW, dry weight.

The first two principal components of 37 growth and physiological traits explained 51.8% and 12.7% of the variation among the 18 treatments, and the first four explained 79.7% (Fig.

12; Supplementary material: Table S10). PC1 contrasted growth and biomass traits (loadings 0.87 to 0.98 for plantlet height, plantlet, root and tuber mass and root and tuber length) with MDA (−0.89), CAT (−0.88), SOD (−0.83) and the days to stages III and IV (−0.64 and −0.71). PC2 was associated mainly with protocorm fresh and dry weight (0.93 and 0.86) and chlorophyll b (0.77). All salt treatments lay on the negative side of PC1, but T18 was displaced towards the growth side and was clearly separated from all other treatments along PC2 (score 6.60).

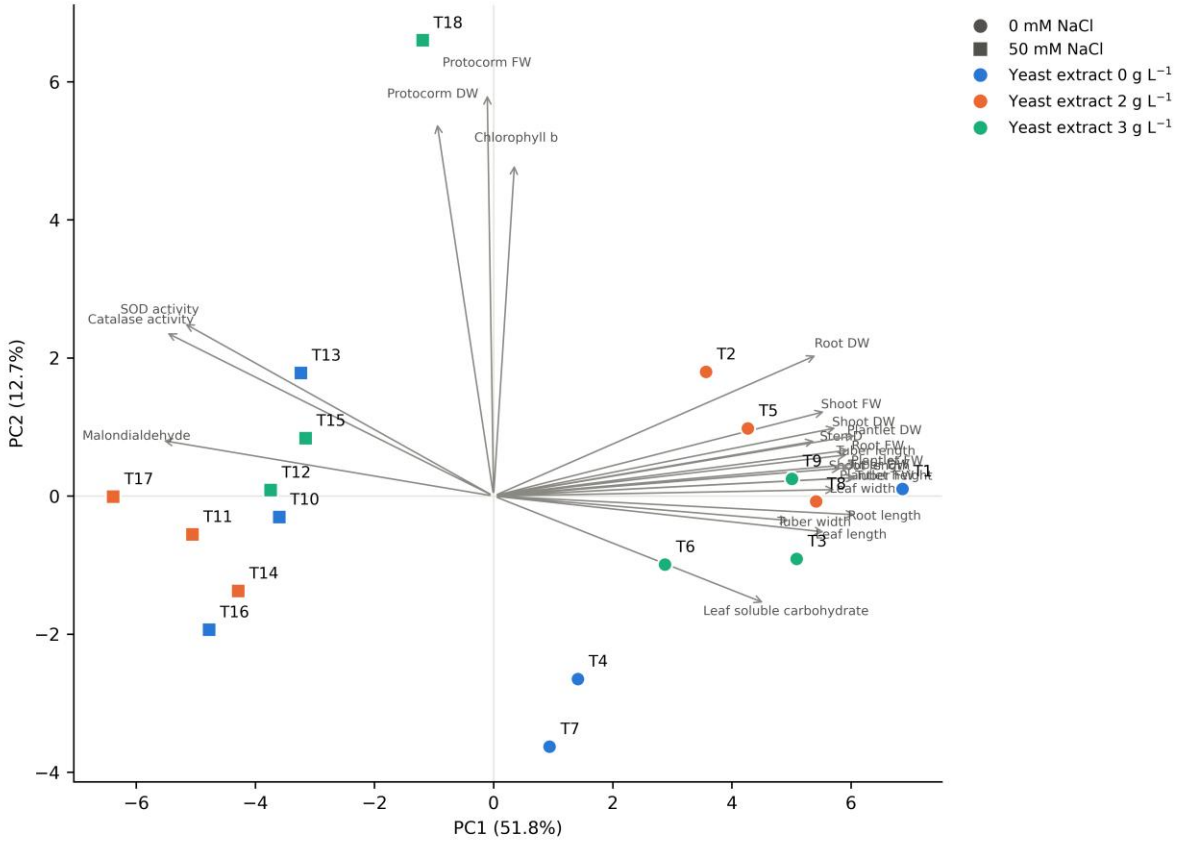


Fig. 12. Principal component analysis. Biplot of the first two principal components based on the standardised means of 37 growth and physiological traits of *A. coriophora* in 18 treatments at 0 and 50 mM NaCl. Circles, 0 mM NaCl; squares, 50 mM NaCl; colours indicate the yeast extract level. Arrows show traits with a vector length of at least 0.75.

Salt tolerance index

The salt tolerance index expressed how much of its unstressed performance each elicitor combination kept at 50 mM NaCl (Table 4; Fig. 13a). Without elicitors, plantlets kept on

average 22 ± 0.5% of their growth and only 7% of their tuber fresh weight. Chitosan alone raised the composite index to 54 ± 1.3% (20 mg L^{-1}) and 56 ± 1.4% (40 mg L^{-1}), and chitosan combined with 3 g L^{-1} yeast extract gave 47 ± 1.1% (20 mg L^{-1}) and 53 ± 1.2% (40 mg L^{-1}), whereas chitosan combined with 2 g L^{-1} yeast extract gave the lowest indices (21 ± 0.5% and 15 ± 0.3%). Germination was much less sensitive than growth, and all combinations kept 80 to 86% of their unstressed germination. Ward's clustering of all 37 traits separated all unstressed from all salt treatments, and T18 formed a separate branch within the salt group (Fig. 13b).

Table 4. Salt tolerance index (STI, %) at 50 mM NaCl for each chitosan × yeast extract (YE) combination

Chitosan (mg L^{-1})	YE (g L^{-1})	Trt (50 mM)	Germi-nation	Plantlet height	Plantlet FW	Root length	Tuber FW	Composite STI
0	0	T10	80 ± 1.0	43 ± 0.4	15 ± 0.8	37 ± 2.4	7 ± 0.6	22 ± 0.5
0	2	T11	82 ± 1.2	69 ± 0.6	23 ± 1.5	55 ± 3.4	13 ± 0.8	34 ± 0.7
0	3	T12	83 ± 1.0	51 ± 0.5	24 ± 1.7	33 ± 1.7	12 ± 1.1	29 ± 0.7
20	0	T13	81 ± 1.2	68 ± 0.7	39 ± 2.6	50 ± 2.8	52 ± 3.8	54 ± 1.3
20	2	T14	83 ± 1.2	48 ± 0.5	18 ± 1.0	42 ± 2.8	6 ± 0.5	21 ± 0.5
20	3	T15	85 ± 1.1	64 ± 0.6	45 ± 3.3	58 ± 3.6	32 ± 1.9	47 ± 1.1
40	0	T16	83 ± 1.2	56 ± 0.5	47 ± 2.4	49 ± 3.1	50 ± 3.3	56 ± 1.4
40	2	T17	85 ± 1.4	33 ± 0.3	12 ± 0.6	20 ± 1.3	5 ± 0.4	15 ± 0.3
40	3	T18	86 ± 1.4	77 ± 0.7	48 ± 3.1	57 ± 3.4	33 ± 2.4	53 ± 1.2

STI = 100 × mean at 50 mM / mean of the same combination at 0 mM NaCl. Values are STI ± SE, with the SE obtained by first-order error propagation (delta method) from the SE of the two means. The composite STI is the mean STI of plantlet height, plantlet fresh and dry weight, root length, and tuber fresh weight, dry weight and length; its SE was propagated from the seven trait SEs. FW, fresh weight.

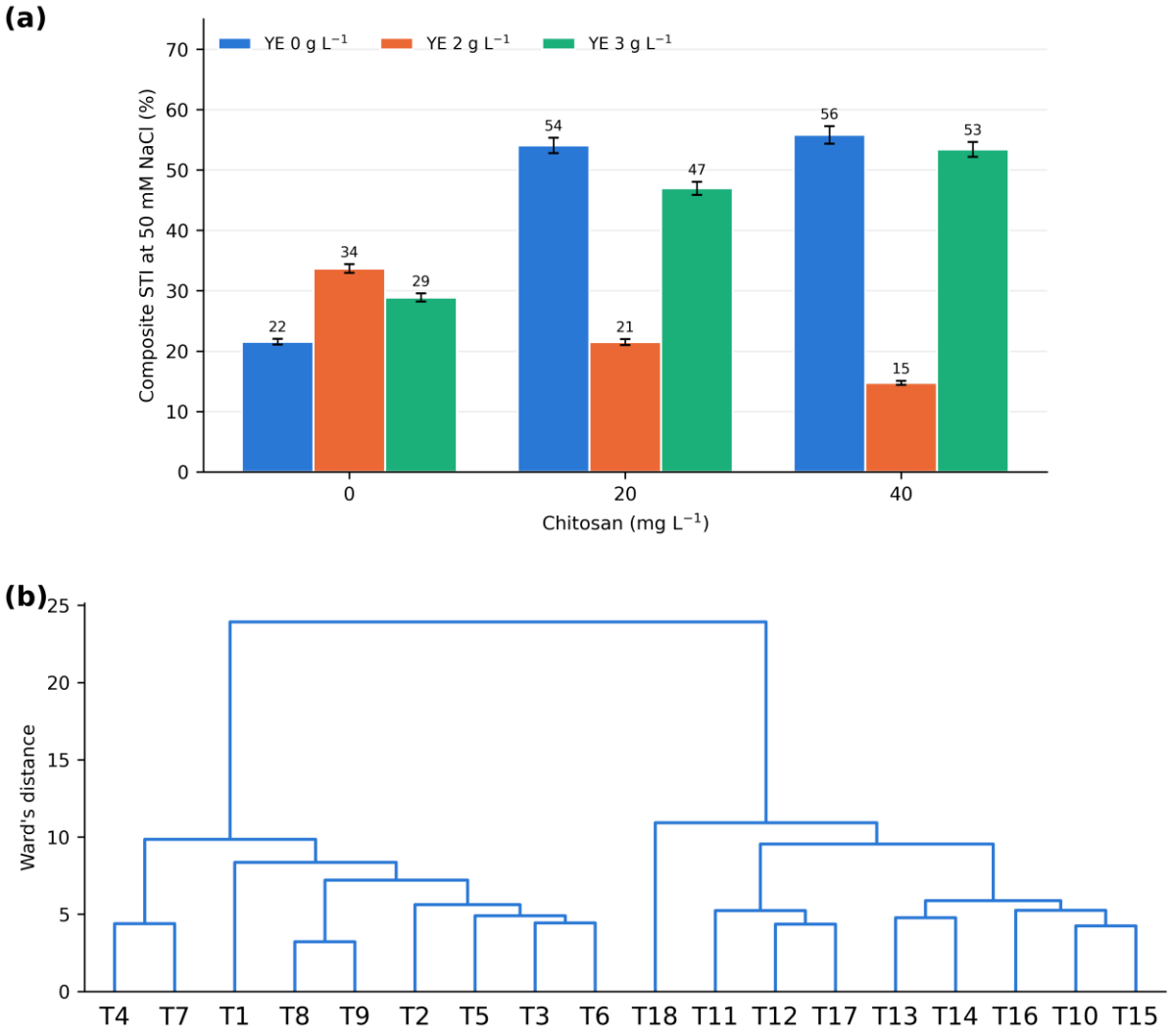


Fig. 13. Salt tolerance and clustering of treatments. (**a**) Composite salt tolerance index (STI) of seven growth and tuber traits at 50 mM NaCl, expressed as a percentage of the same chitosan × yeast extract (YE) combination at 0 mM NaCl; bars are STI ± SE obtained by error propagation. (**b**) Dendrogram of the 18 treatments at 0 and 50 mM NaCl obtained by Ward's clustering of the standardized means of 37 traits.

Discussion

Anacamptis coriophora is sensitive to salinity from germination onwards

The slower and lower germination under NaCl is consistent with the general response of seeds to salinity, in which the osmotic effect of salt limits imbibition and ion toxicity damages the embryo at higher concentrations [26, 46]. Orchid seeds are especially exposed because the embryo is covered only by a thin testa and has almost no reserves to buffer stress [7, 14, 47]. The germination timeline showed that the strongest delay occurred at the rhizoid stage. Rhizoids

are the absorptive structures through which protocorms take up water and nutrients, and in nature they are also the main entry point for mycorrhizal hyphae [13, 47]. A salt-induced delay at this point slows the transition from a reserve-dependent embryo to a protocorm that can feed itself. Once rhizoids had formed, however, protocorms developed faster under salt. We think the embryo kept accumulating material while rhizoid emergence was held back. Stage-based scoring of this kind has been used to compare media and treatments in other terrestrial orchids [18–20]. In our case it showed that salinity acts mainly on the early steps of development.

Germination itself was relatively tolerant, with 80 to 86% of the unstressed value retained at 50 mM, but plantlet growth and particularly tuber formation were much more sensitive. The complete lack of tubers at 100 mM NaCl and the loss of about 98% of biomass place *A. coriophora* among salt-sensitive plants, in agreement with its natural habitat in damp, non-saline meadows. The strong reduction of shoot growth at 50 mM is typical of the osmotic phase of salt stress, in which leaf expansion is restricted long before ions reach toxic levels [27, 48], whereas the near-arrest of growth at 100 mM suggests that ionic effects were also involved [26]. NaCl alone explained more than 90% of the variation in plantlet biomass, so there was little room for any other factor to compensate. Tissue culture studies rarely report effect sizes, yet they help to tell statistically significant effects from those that matter in practice [49].

The larger protocorms formed under salt most likely reflect a slower transition from protocorm to plantlet rather than better growth. The protocorm is a transient organ that feeds the developing shoot and root and shrinks as the young plant becomes autotrophic [47], and the retention of carbon in sink tissues when shoot expansion is limited is a common response to salinity [27, 50]. The simultaneous rise of MDA, CAT and SOD shows that 50 mM NaCl caused oxidative stress and that the antioxidant enzymes responded, but not enough to prevent membrane damage. This pattern is common in salt-sensitive tissues [51, 52]. The loss of chlorophyll a and especially carotenoids, the decline of leaf soluble carbohydrates and the

modest rise of proline are consistent with this. In many plants soluble sugars accumulate in leaves under salinity [50], and their decline here suggests that reduced photosynthesis limited sugar supply more than osmotic adjustment raised it. Proline was highest in a treatment that did not grow well (T11) and was unrelated to plantlet biomass in the correlation analysis, which supports the view that high proline often marks stress severity rather than causing tolerance [53, 54].

Correlations after removing the salt effect

At first sight, the correlation matrix showed every growth trait falling as oxidative damage and antioxidant enzyme activity rose. Most of these correlations disappeared when the NaCl effect was removed. They arose because salinity moved all traits at once, not because the traits influenced one another. Pooling treatment means from a factorial experiment carries this risk [55]. The associations that survived the correction tell us more. The close coupling of plantlet, root and tuber mass within each salinity level indicates that tuber size in vitro is limited by the size of the plant that feeds it, so that any treatment that improves shoot and root growth should also improve tubers. The persistent link between chlorophyll b and protocorm fresh weight, which was unchanged by the correction, suggests a shared response of these two traits to the elicitors rather than to salt. The CAT and MDA relationship changed sign. Across salinity levels both rose together, but within a level the treatments with higher CAT activity had less lipid peroxidation. This would be expected if CAT, besides being induced by salt, actually limited membrane damage [56], and it agrees with studies of salt-stressed strawberry in which treatments that strengthened the antioxidant system reduced lipid peroxidation [29–31].

Elicitors offered no advantage without salt

Neither chitosan nor yeast extract improved germination or biomass relative to the untreated control on P723 medium, and chitosan alone reduced plantlet and tuber mass.

Chitosan effects in orchids are dose-dependent and species-specific: it promoted germination in one *Dendrobium* species but not in another [39], and its effect on *Serapias vomeracea* depended on the chitosan form [40]. Activation of defense signaling in the absence of stress can impose a cost on growth [57], and the poor germination with the higher chitosan dose combined with yeast extract fits a hormetic pattern in which higher elicitor doses become inhibitory [58]. Biostimulants such as salicylic acid and simvastatin have likewise given treatment-specific responses in savory cultured in vitro [23]. Chitosan alone also increased leaf soluble carbohydrates while reducing growth, which points to carbon being available but not used for expansion, as expected when defense responses are activated [57]. Yeast extract sharply increased chlorophyll content even without salt and was the main source of variance in all chlorophyll traits, most likely because it supplies organic nitrogen, amino acids and vitamins needed for pigment synthesis [43, 45]. The germination of 86% on unamended P723 medium is high for a temperate terrestrial orchid, whose germination is often low and variable [17, 59], and confirms that this medium is well suited to *A. coriophora*. Asymbiotic protocols have also proved effective for other Iranian salep orchids [19–21].

Chitosan combined with yeast extract mitigated moderate salinity

Under 50 mM NaCl the elicitors had clear benefits. The lower chitosan dose accelerated protocorm formation, which suggests a priming effect similar to that seen in chitosan-primed ajowan seeds under salinity, where higher antioxidant activity and lower lipid peroxidation were found [60]. Seed priming against salt stress often works this way [46]. For plantlet growth, however, the combination of 40 mg L^{-1} chitosan with 3 g L^{-1} yeast extract (T18) worked best. It tripled plantlet fresh weight, increased root length by half and tuber fresh weight almost fivefold compared with the stressed control. The multivariate analyses gave the same answer: T18 had the highest PC2 score, formed its own branch in both cluster analyses and was the only combination that joined a high tolerance index with high absolute growth. Chitosan alone gave

similar tolerance indices, but largely because it reduced growth in the absence of salt, so that the relative loss under salt was smaller. A tolerance index therefore needs to be read alongside absolute performance under stress [48]. The standard errors of the index were small, so the differences among combinations were much larger than the uncertainty of each ratio.

Several mechanisms may have contributed to the effect of T18. Both elicitors can trigger a moderate production of reactive oxygen species that acts as a signal for protective genes [34, 61], so that plantlets exposed to the elicitors may respond more rapidly when salt stress develops; this is how chemical priming is thought to work [62]. Chitosan has been shown to reduce Na^+ accumulation, increase compatible solutes and polyamines and protect membranes and the photosynthetic apparatus under salinity [41, 42, 63], and the very high chlorophyll content and low MDA of T18 are consistent with such protection. Yeast extract supplies amino acids and vitamins that can support the synthesis of proteins and protective compounds under stress [43, 45], and its significant effect on SOD indicates that it also supported the antioxidant system. The unusual pigment composition of T18, with very high chlorophyll b and very low carotenoids, may reflect an increase in light-harvesting antenna relative to reaction centers [64], although this needs to be confirmed. The benefit was specific to the dose combination. T17, which differed only by 1 g L^{-1} of yeast extract, was the poorest treatment at 50 mM, and at 100 mM the T18 combination gave the smallest plantlets and no roots. The first result suggests that the balance between the nitrogen supplied by yeast extract, the peptone already present in P723 and the defense stimulus of chitosan matters, and terrestrial orchids are known to be sensitive to the form and amount of nitrogen [65]. The large chitosan × yeast extract interaction for proline and chlorophyll supports this idea. The second agrees with the hormetic model, in which the benefit of a stimulus disappears when the total stress load becomes too high [58].

Implications for conservation and reintroduction

These results have some practical consequences for *A. coriophora* and other salep orchids. Under optimal conditions, unamended P723 medium gave the best germination and good growth, so elicitors are not needed for routine propagation. Where they may help is in producing plants that will face stress, for example when they are destined for reintroduction into degraded or slightly saline sites, where survival after transfer is often the weakest link of orchid reintroduction programs [16]. A pre-conditioning phase with 40 mg L^{-1} chitosan and 3 g L^{-1} yeast extract could be tested for this purpose, provided that its benefit persists after transfer to soil. At the same time, the strong salt sensitivity of the species, and in particular the complete suppression of tuber formation at 100 mM NaCl, indicate that reintroduction sites should be selected in non-saline meadows and that irrigation water with appreciable salt content should be avoided in any cultivation attempt. Because salep orchid populations are genetically differentiated [8, 10, 11], seed for such programs should come from several local populations, and their salt tolerance may differ from that of the single seed lot used here.

Our study has some limitations. Physiological traits were measured on three replicates, which limited the power to detect interactions, as seen for MDA, CAT and SOD, where the residual accounted for up to a third of the variation. The correlation and multivariate analyses were based on 18 treatment means and describe associations among treatments, not among individual plants. The 100 mM level could not be included in the physiological analyses, the ionic composition of the tissues was not measured, and the plantlets have not yet been acclimatized, so their performance in soil, their mycorrhizal colonization and their tuber development in later seasons remain to be tested.

Conclusions

Anacamptis coriophora germinated reasonably well at 50 mM NaCl, but rhizoid formation was strongly delayed, plantlet growth and tuber formation were sharply reduced at

this level, and 100 mM NaCl almost stopped growth and completely prevented tuberization. Salinity induced oxidative stress, shown by the rise of MDA together with CAT and SOD, and once the salt effect was removed, higher CAT activity was associated with less lipid peroxidation. Without salt, neither chitosan nor yeast extract improved germination or growth, although yeast extract increased chlorophyll content. Under moderate salinity, 20 mg L^{-1} chitosan accelerated protocorm formation, and the combination of 40 mg L^{-1} chitosan with 3 g L^{-1} yeast extract restored a large part of the lost plantlet, root and tuber growth. The tolerance index, principal component and cluster analyses all pointed to this treatment. This combination is a promising candidate for pre-conditioning plantlets destined for reintroduction, and its long-term effect after transfer to soil should be the focus of future work.

Methods

Plant material and seed sterilization

Mature, undehisced capsules of *A. coriophora* were collected from a natural population near Salian village, Kurdistan province, western Iran (35°15′19.6″ N, 46°38′26.6″ E; 1737 m a.s.l.), with the permission of the Natural Resources and Watershed Management Organization and the Department of Environment, Sanandaj. The species was authenticated by Eng. Hossein Maroofi (Agriculture and Natural Resources Research Center, Sanandaj, Iran), and a voucher specimen was deposited in the herbarium of the University of Kurdistan, Sanandaj, Iran. Capsules were dried at room temperature, and the seeds were stored in paper envelopes in a desiccator at 4 °C until sowing. Seeds were surface-sterilized in 0.4% (w/v) sodium hypochlorite for 10 to 15 min, following a protocol standardized for tuberous terrestrial orchids [19], with the change of seed color from brown to white used as the end point. The seeds were not rinsed and were spread on the medium with a sterile loop and spreader.

Experimental design and treatments

Two NaCl levels, 50 and 100 mM, were selected to represent moderate and severe salinity for glycophytes [27, 48]. The experiment was set up as a 3 × 3 × 3 factorial in a completely randomized design with NaCl (0, 50 and 100 mM), chitosan (0, 20 and 40 mg L^{-1}) and yeast extract (0, 2 and 3 g L^{-1}). The 27 treatments were numbered T1 to T27, with T1 to T9 at 0 mM, T10 to T18 at 50 mM and T19 to T27 at 100 mM NaCl. Within each salinity level, treatments were ordered by chitosan and then by yeast extract (for example, T10, T11 and T12 received 0 mg L^{-1} chitosan with 0, 2 and 3 g L^{-1} yeast extract; T18 received 40 mg L^{-1} chitosan and 3 g L^{-1} yeast extract). Each culture vessel was an experimental unit. Five vessels per treatment were used for germination percentage and morphological traits, and three for the timing of germination stages and for physiological traits. Low-molecular-weight chitosan (Sigma-Aldrich, St. Louis, MO, USA; product no. 448869; 50 to 190 kDa; 75 to 85% deacetylation) was dissolved in 1% (v/v) acetic acid to give a 1 g L^{-1} stock solution, whose pH was raised to 5.8 with 1 M NaOH. The chitosan stock and yeast extract were added to the medium before the agar.

Culture medium and incubation

Seeds were sown on Orchid Seed Sowing Medium P723 (PhytoTechnology Laboratories, Lenexa, KS, USA), to which 20 g L^{-1} sucrose and 1 g L^{-1} activated charcoal were added. Chitosan, yeast extract and NaCl were added according to the treatment, the pH was adjusted to 5.75 and the medium was solidified with 8 g L^{-1} agar. The electrical conductivity (EC) of the basal medium at 25 °C, before sucrose and agar were added, was about 1.5 dS m^{-1}. Based on the molar conductivity of NaCl at 25 °C (about 106 to 111 S cm^2 mol^{-1}), 50 and 100 mM NaCl added about 5.2 and 10.1 dS m^{-1}, so that the media reached about 6.7 and 11.6 dS m^{-1}, respectively. Media were autoclaved at 121 °C for 15 min and dispensed at 30 mL per vessel. Cultures were kept in a growth chamber at 25 ± 1 °C, in darkness until protocorms formed and

545 then under a 16/8 h light/dark photoperiod at a photosynthetic photon flux density of 40 µmol
546 m^{-2} s^{-1}. The positions of the vessels were re-randomized every week.

547 **Germination and growth measurements**

548 The number of days needed to reach six developmental stages was recorded for each
549 vessel: stage I, no germination; stage II, swelling of the embryo; stage III, rupture of the testa;
550 stage IV, rhizoid formation; stage V, a well-formed protocorm; and stage VI, emergence of the
551 first shoot (Fig. 1), following the logic of stage systems used for terrestrial orchids [18, 66].
552 Final germination percentage was the proportion of sown seeds that reached stage V. When
553 plantlets in the control had formed roots and small tubers, plantlet height, protocorm length and
554 width, stem diameter, leaf number, leaf length and width, root number and length, tuber number,
555 length and width, and shoot length were measured with a digital calliper or under a
556 stereomicroscope. Fresh weights of whole plantlets, shoots, roots, tubers and protocorms were
557 recorded immediately, and dry weights after drying at 70 °C to constant weight.

558 **Physiological assays**

559 Leaves from all plantlets in a vessel were pooled to form one replicate sample.
560 Chlorophyll a, chlorophyll b and total chlorophyll were determined in 80% acetone extracts
561 [67] and carotenoids according to Lichtenthaler [64]. Free proline was measured with acid
562 ninhydrin at 520 nm [68] and expressed as µg g^{-1} fresh weight (FW). Leaf soluble carbohydrates
563 were extracted in 80% (v/v) ethanol, determined with the phenol and sulfuric acid method at
564 490 nm against a glucose standard [69] and expressed as µg g^{-1} FW. Lipid peroxidation was
565 estimated as malondialdehyde (MDA) with the thiobarbituric acid reaction [70], corrected for
566 interfering compounds [71], using an extinction coefficient of 155 mM^{-1} cm^{-1}, and expressed
567 as nmol g^{-1} FW. For enzyme assays, 0.5 g of fresh leaf tissue was ground in liquid nitrogen and
568 homogenized in 3 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1

569 mM EDTA and 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000 ×
570 g for 20 min at 4 °C, and the supernatant was used for the enzyme and protein assays. Catalase
571 (CAT) activity was measured in 50 mM potassium phosphate buffer (pH 7.0) containing 10
572 mM H_2O_2 as the decrease in absorbance at 240 nm over 1 min ($\varepsilon = 39.4\ mM^{-1}\ cm^{-1}$), and one
573 unit (U) was defined as the decomposition of 1 μmol $H_2O_2\ min^{-1}$ [72]. Superoxide dismutase
574 (SOD) activity was assayed in 50 mM potassium phosphate buffer (pH 7.8) containing 13 mM
575 methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin and 0.1 mM EDTA; tubes
576 were illuminated for 15 min and absorbance was read at 560 nm, and one unit was defined as
577 the amount of enzyme that inhibited NBT photoreduction by 50% [73]. Soluble protein was
578 determined by the Bradford method with bovine serum albumin as the standard [74], and
579 enzyme activities were expressed as $U\ mg^{-1}$ protein.

580 **Statistical analysis**

581 At 100 mM NaCl, the plantlets formed no tubers, formed no roots in the three treatments
582 with 40 mg L^{-1} chitosan (T25 to T27) and did not yield enough tissue for physiological assays.
583 Because this missing level was not random, the data were analyzed in two tiers. Traits observed
584 in all 27 treatments (germination stages, germination percentage, protocorm, shoot and plantlet
585 biomass traits) were analyzed as a 3 × 3 × 3 factorial. Root, tuber and physiological traits were
586 analyzed as a 2 × 3 × 3 factorial using the 18 treatments at 0 and 50 mM NaCl, and the 100 mM
587 level was described separately. Normality of residuals and homogeneity of variances were
588 checked with the Shapiro–Wilk and Levene tests [75], and four biomass traits with
589 heterogeneous variances (plantlet, shoot and tuber fresh weight and plantlet dry weight) were
590 analyzed after $\log_{10}$ transformation; untransformed means are presented. All means are given
591 with their standard error (SE). Means were compared with Duncan's multiple range test at $P \leq$
592 0.05 [55, 76]. When the three-way interaction was significant, the treatment means were
593 compared; otherwise the significant lower-order effects were presented. Tukey's test was

computed as a check and is given in Supplementary material (Table S5). The full analysis of variance tables are given in Supplementary material (Tables S1 to S4).

The contribution of each source of variation was expressed as its share of the total sum of squares, an eta-squared type effect size [49]. A salt tolerance index (STI) was calculated for each trait and each chitosan × yeast extract combination as STI (%) = 100 × (mean at 50 mM NaCl)/(mean at 0 mM NaCl). Its standard error was obtained by first-order error propagation (delta method) as $SE(STI) = STI \times [(SE_{50}/mean_{50})^2 + (SE_0/mean_0)^2]^{0.5}$. A composite index was calculated as the mean STI of seven growth traits (plantlet height, plantlet fresh and dry weight, root length, and tuber fresh weight, dry weight and length), with its SE propagated from the trait SEs.

Relationships among traits were examined on the standardized means of the 18 treatments at 0 and 50 mM NaCl. Pearson correlation coefficients were calculated among 25 traits. To separate associations caused by the common response to salinity from those that held within a salinity level, partial correlations were calculated after removing the NaCl effect, by correlating the deviations of each treatment mean from the mean of its salinity level (df = 15). The false discovery rate was controlled by the Benjamini–Hochberg procedure [77]. Two-way hierarchical clustering of treatments and traits used Ward's method on Euclidean distances [78], and principal component analysis (PCA) was performed on 37 traits [79]. Analyses were run in Python with statsmodels, SciPy, scikit-learn and seaborn [80, 81].

Abbreviations

CAT: Catalase; DW: Dry weight; FW: Fresh weight; MDA: Malondialdehyde; PCA: Principal component analysis; SE: Standard error; SOD: Superoxide dismutase; STI: Salt tolerance index; YE: Yeast extract

Supplementary Information

Supplementary material. Tables S1 to S10 (Excel workbook). Table S1, analysis of variance of germination, protocorm and plantlet traits (3 × 3 × 3 factorial); Table S2, analysis of variance of root, tuber and physiological traits (2 × 3 × 3 factorial); Table S3, full ANOVA output for all traits; Table S4, root and tuber formation at 100 mM NaCl; Table S5, treatment means ± SE with Duncan and Tukey groupings for all traits; Table S6, main-effect and two-way means ± SE; Table S7, salt tolerance index ± SE of individual traits; Table S8, effect sizes; Table S9, Pearson and partial correlation matrices with P values; Table S10, principal component loadings and scores.

Declarations

Ethics approval and consent to participate: Seed capsules of *Anacamptis coriophora* were collected from a wild population with the permission of the Natural Resources and Watershed Management Organization and the Department of Environment, Sanandaj, Iran. The collection and use of plant material complied with relevant institutional, national and international guidelines and legislation, including the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the IUCN Policy Statement on Research Involving Species at Risk of Extinction. Only capsules were collected, and no plants were removed.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files. Raw data and analysis scripts are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: NOA performed the experiments, collected the data and wrote the first draft. YV conceived and designed the study, supervised the work, analyzed the data and revised the manuscript. JK and AAM contributed to the experimental design and the interpretation of the results and revised the manuscript. All authors read and approved the final manuscript.

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References

1. Chase MW, Cameron KM, Freudenstein JV, Pridgeon AM, Salazar G, van den Berg C, Schuiteman A. An updated classification of Orchidaceae. Bot J Linn Soc. 2015;177(2):151–74.

2. Swarts ND, Dixon KW. Terrestrial orchid conservation in the age of extinction. Ann Bot. 2009;104(3):543–56.

3. Hinsley A, de Boer HJ, Fay MF, Gale SW, Gardiner LM, Gunasekara RS, Kumar P, Masters S, Metusala D, Roberts DL, Veldman S, Wong S, Phelps J. A review of the trade in orchids and its implications for conservation. Bot J Linn Soc. 2018;186(4):435–55.

4. Kasparek M, Grimm U. European trade in Turkish salep with special reference to Germany. Econ Bot. 1999;53(4):396–406.

5. Li QY, Dou ZM, Chen C, Jiang YM, Yang B, Fu X. Study on the effect of molecular weight on the gut microbiota fermentation properties of blackberry polysaccharides in vitro. J Agric Food Chem. 2022;70(36):11245–57.

6. Ghorbani A, Gravendeel B, Naghibi F, de Boer H. Wild orchid tuber collection in Iran: A wake-up call for conservation. Biodivers Conserv. 2014;23(11):2749–60.

7. Vafaee Y, Mohammadi G, Nazari F, Fatahi M, Kaki A, Gholami S, Ghorbani A, Khadivi A. Phenotypic characterization and seed-micromorphology diversity of the threatened terrestrial orchids: Implications for conservation. S Afr J Bot. 2021;137:386–98.

8. Gholami S, Vafaee Y, Nazari F, Ghorbani A. Molecular characterization of endangered Iranian terrestrial orchids using ISSR markers and association with floral and tuber-related phenotypic traits. Physiol Mol Biol Plants. 2021;27(1):53–68.

9. Kaki A, Vafaee Y, Khadivi A. Genetic variation of *Anacamptis coriophora*, *Dactylorhiza umbrosa*, *Himantoglossum affine*, *Orchis mascula*, and *Ophrys schulzei* in the western parts of Iran. Ind Crops Prod. 2020;156:112854.

10. Gholami S, Vafaee Y, Nazari F, Ghorbani A. Exploring genetic variations in threatened medicinal orchids using start codon targeted (SCoT) polymorphism and marker-association with seed morphometric traits. Physiol Mol Biol Plants. 2021;27(4):769–85.

11. Palaz EB, Demirel F, Adali S, Demirel S, Yilmaz A. Genetic relationships of salep orchid species and gene flow among *Serapias vomeracea* × *Anacamptis morio* hybrids. Plant Biotechnol Rep. 2023;17(2):315–27.

12. Bateman RM, Hollingsworth PM, Preston J, Luo YB, Pridgeon AM, Chase MW. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). Bot J Linn Soc. 2003;142(1):1–40.

685 13. Rasmussen HN. Terrestrial orchids: from seed to mycotrophic plant. Cambridge:
686 Cambridge University Press; 1995.
687 14. Arditti J, Ghani AKA. Numerical and physical properties of orchid seeds and their
688 biological implications. New Phytol. 2000;145(3):367–421.
689 15. Kauth PJ, Dutra D, Johnson TR, Stewart SL, Kane ME, Vendrame W. Techniques and
690 applications of in vitro orchid seed germination. In: Teixeira da Silva JA, editor.
691 Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues. Vol. 5.
692 Isleworth: Global Science Books; 2008. p. 375–91.
693 16. Reiter N, Whitfield J, Pollard G, Bedggood W, Argall M, Dixon K, Davis B, Swarts N.
694 Orchid re-introductions: An evaluation of success and ecological considerations using key
695 comparative studies from Australia. Plant Ecol. 2016;217(1):81–95.
696 17. Van Waes JM, Debergh PC. In vitro germination of some Western European orchids.
697 Physiol Plant. 1986;67(2):253–61.
698 18. Stewart SL, Kane ME. Asymbiotic seed germination and in vitro seedling development of
699 *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell
700 Tissue Organ Cult. 2006;86(2):147–58.
701 19. Fatahi M, Vafaee Y, Nazari F, Tahir NAR. In vitro asymbiotic seed germination,
702 protocorm formation, and plantlet development of *Orchis simia* Lam.: A threatened
703 terrestrial orchid species. S Afr J Bot. 2022;151:156–65.
704 20. Fatahi M, Anghelescu NE, Vafaee Y, Khoddamzadeh AA. Micropropagation of
705 *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski through asymbiotic seed germination and
706 somatic embryogenesis: a promising tool for conservation of rare terrestrial orchids. S Afr
707 J Bot. 2023;159:492–506.

21. Fatahi M, Vafaee Y, Tahir NA, Khorshidi J. Large-scale in vitro multiplication and phytochemical analysis of *Himantoglossum affine* (Boiss.) Schltr.: an endangered Euro-Mediterranean terrestrial orchid. Diversity. 2022;14(12):1137.

22. Mozafari AA, Vafaee Y, Karami E. In vitro propagation and conservation of *Satureja avromanica* Maroofi: an indigenous threatened medicinal plant of Iran. Physiol Mol Biol Plants. 2015;21(3):433–9.

23. Noori N, Mozafari AA, Khorshidi J. Biostimulants effect of salicylic acid, simvastatin and indole butyric acid on regeneration and some of morphological and physiological characteristics of *Satureja avromanica* Maroofi under in vitro conditions. J Plant Biol Sci. 2020;11:19–38.

24. Bektaş E, Cüce M, Sökmen A. In vitro germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. Turk J Bot. 2013;37(2):336–42.

25. Hassani A, Azapagic A, Shokri N. Global predictions of primary soil salinization under changing climate in the 21st century. Nat Commun. 2021;12:6663.

26. Munns R. Comparative physiology of salt and water stress. Plant Cell Environ. 2002;25(2):239–50.

27. Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 2008;59:651–81.

28. Mozafari AA, Ghadakchi asl A, Ghaderi N. Grape response to salinity stress and role of iron nanoparticle and potassium silicate to mitigate salt induced damage under in vitro conditions. Physiol Mol Biol Plants. 2018;24(1):25–35.

29. Golnari S, Vafaee Y, Nazari F, Ghaderi N. Gamma-aminobutyric acid (GABA) and salinity impacts antioxidative response and expression of stress-related genes in strawberry cv. Aromas. Braz J Bot. 2021;44:639–51.

30. Dedejani S, Mozafari AA, Ghaderi N. Salicylic acid and iron nanoparticles application to mitigate the adverse effects of salinity stress under in vitro culture of strawberry plants. Iran J Sci Technol Trans A Sci. 2021;45(3):821–31.

31. Moradi P, Vafaee Y, Mozafari AA, Tahir NA. Silicon nanoparticles and methyl jasmonate improve physiological response and increase expression of stress-related genes in strawberry cv. Paros under salinity stress. Silicon. 2022;14:10559–69.

32. Arditti J. Factors affecting the germination of orchid seeds. Bot Rev. 1967;33(1):1–97.

33. Abdullakasim S, Kongpaisan P, Thongjang P, Saradhuldhat P. Physiological responses of potted *Dendrobium* orchid to salinity stress. Hortic Environ Biotechnol. 2018;59(4):491–8.

34. Zhao J, Davis LC, Verpoorte R. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv. 2005;23(4):283–333.

35. Ahmadi Soleimanie S, Vafaee Y, Koushesh Saba M. Preharvest application of sodium nitroprusside improves tomato fruit quality and alleviates chilling injury during cold storage. Int J Veg Sci. 2020;26:364–78.

36. Yaghoobi K, Ghaderi N, Vafaee Y, Javadi T. Potassium silicate alleviates deleterious effects of salinity on two strawberry cultivars grown under soilless pot culture. Sci Hortic. 2016;213:87–95.

37. Safari S, Nazari F, Vafaee Y, Teixeira da Silva JA. Impact of rice husk biochar on drought stress tolerance in perennial ryegrass (*Lolium perenne* L.). J Plant Growth Regul. 2023;42:810–26.

38. Nge KL, Nwe N, Chandrkrachang S, Stevens WF. Chitosan as a growth stimulator in orchid tissue culture. Plant Sci. 2006;170(6):1185–90.

39. Kananont N, Pichyangkura R, Chanprame S, Chadchawan S, Limpanavech P. Chitosan specificity for the in vitro seed germination of two *Dendrobium* orchids (Asparagales: Orchidaceae). Sci Hortic. 2010;124(2):239–47.

40. Acemi A. Chitosan versus plant growth regulators: A comparative analysis of their effects on in vitro development of *Serapias vomeracea* (Burm.f.) Briq. Plant Cell Tissue Organ Cult. 2020;141(2):327–38.

41. Geng W, Li Z, Hassan MJ, Peng Y. Chitosan regulates metabolic balance, polyamine accumulation, and Na+ transport contributing to salt tolerance in creeping bentgrass. BMC Plant Biol. 2020;20:506.

42. Zhang G, Wang Y, Wu K, Zhang Q, Feng Y, Miao Y, Yan Z. Exogenous application of chitosan alleviate salinity stress in lettuce (*Lactuca sativa* L.). Horticulturae. 2021;7(10):342.

43. Utami ESW, Hariyanto S. Organic compounds: Contents and their role in improving seed germination and protocorm development in orchids. Int J Agron. 2020;2020:2795108.

44. Baenas N, García-Viguera C, Moreno DA. Elicitation: A tool for enriching the bioactive composition of foods. Molecules. 2014;19(9):13541–63.

45. Hammad SAR, Ali OAM. Physiological and biochemical studies on drought tolerance of wheat plants by application of amino acids and yeast extract. Ann Agric Sci. 2014;59(1):133–45.

46. Ibrahim EA. Seed priming to alleviate salinity stress in germinating seeds. J Plant Physiol. 2016;192:38–46.

47. Yeung EC. A perspective on orchid seed and protocorm development. Bot Stud. 2017;58:33.

48. Negrão S, Schmöckel SM, Tester M. Evaluating physiological responses of plants to salinity stress. Ann Bot. 2017;119(1):1–11.

49. Olejnik S, Algina J. Generalized eta and omega squared statistics: Measures of effect size for some common research designs. Psychol Methods. 2003;8(4):434–47.

50. Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, Prado FE. Soluble sugars: Metabolism, sensing and abiotic stress. Plant Signal Behav. 2009;4(5):388–93.

51. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 2010;48(12):909–30.

52. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J Bot. 2012;2012:217037.

53. Szabados L, Savouré A. Proline: A multifunctional amino acid. Trends Plant Sci. 2010;15(2):89–97.

54. Kavi Kishor PB, Sreenivasulu N. Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant Cell Environ. 2014;37(2):300–11.

55. Gomez KA, Gomez AA. Statistical procedures for agricultural research. 2nd ed. New York: John Wiley & Sons; 1984.

56. Apel K, Hirt H. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 2004;55:373–99.

57. Huot B, Yao J, Montgomery BL, He SY. Growth–defense tradeoffs in plants: A balancing act to optimize fitness. Mol Plant. 2014;7(8):1267–87.

58. Agathokleous E, Kitao M, Calabrese EJ. Hormesis: A compelling platform for sophisticated plant science. Trends Plant Sci. 2019;24(4):318–27.

59. Ponert J, Vosolsobě S, Kmecová K, Lipavská H. European orchid cultivation: From seed to mature plant. Eur J Environ Sci. 2011;1(2):95–107.

60. Mahdavi B, Rahimi A. Seed priming with chitosan improves the germination and growth performance of ajowan (*Carum copticum*) under salt stress. EurAsian J Biosci. 2013;7:69–76.

61. Mittler R. ROS are good. Trends Plant Sci. 2017;22(1):11–9.

62. Savvides A, Ali S, Tester M, Fotopoulos V. Chemical priming of plants against multiple abiotic stresses: Mission possible? Trends Plant Sci. 2016;21(4):329–40.

63. Yang F, Hu J, Li J, Wu X, Qian Y. Chitosan enhances leaf membrane stability and antioxidant enzyme activities in apple seedlings under drought stress. Plant Growth Regul. 2009;58(2):131–6.

64. Lichtenthaler HK. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. 1987;148:350–82.

65. Ponert J, Figura T, Vosolsobě S, Lipavská H, Vohník M, Jersáková J. Asymbiotic germination of mature seeds and protocorm development of *Pseudorchis albida* (Orchidaceae) are inhibited by nitrates even at extremely low concentrations. Botany. 2013;91(10):662–70.

66. Dutra D, Johnson TR, Kauth PJ, Stewart SL, Kane ME, Richardson L. Asymbiotic seed germination, in vitro seedling development, and greenhouse acclimatization of the threatened terrestrial orchid *Bletia purpurea*. Plant Cell Tissue Organ Cult. 2008;94(1):11–21.

824 67. Arnon DI. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*.
825 Plant Physiol. 1949;24(1):1–15.
826 68. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress
827 studies. Plant Soil. 1973;39(1):205–7.
828 69. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for
829 determination of sugars and related substances. Anal Chem. 1956;28(3):350–6.
830 70. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: I. Kinetics and
831 stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 1968;125(1):189–98.
832 71. Hodges DM, DeLong JM, Forney CF, Prange RK. Improving the thiobarbituric acid-
833 reactive-substances assay for estimating lipid peroxidation in plant tissues containing
834 anthocyanin and other interfering compounds. Planta. 1999;207(4):604–11.
835 72. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121–6.
836 73. Giannopolitis CN, Ries SK. Superoxide dismutases: I. Occurrence in higher plants. Plant
837 Physiol. 1977;59(2):309–14.
838 74. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities
839 of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1–
840 2):248–54.
841 75. Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples).
842 Biometrika. 1965;52(3–4):591–611.
843 76. Duncan DB. Multiple range and multiple F tests. Biometrics. 1955;11(1):1–42.
844 77. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful
845 approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995;57(1):289–300.
846 78. Ward JH. Hierarchical grouping to optimize an objective function. J Am Stat Assoc.
847 1963;58(301):236–44.

79. Jolliffe IT, Cadima J. Principal component analysis: A review and recent developments. Philos Trans A Math Phys Eng Sci. 2016;374(2065):20150202.

80. Seabold S, Perktold J. Statsmodels: econometric and statistical modeling with Python. In: Proceedings of the 9th Python in Science Conference. Austin; 2010. p. 92–6.

81. Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, et al. SciPy 1.0: Fundamental algorithms for scientific computing in Python. Nat Methods. 2020;17(3):261–72

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