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Acetic acid disturbs rice germination and post-germination under controlled conditions mimicking green mulching in flooded paddy

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Highlights

- *Based on genotype, increasing acetic acid levels in flooding waters can speed rice germination.*
- *Roots are more sensitive than shoots to acetic acid phytotoxicity during very early germination.*
- *Early root length impairments provide information on tolerance to acetic acid phytotoxicity.*
- *A slower germination rate may induce higher tolerance to green mulching.*

Abstract

Cover crop use in organic rice cropping systems efficiently manages the two most limiting factors in organic agriculture - weed competition and nutrient availability. Nonetheless, cover crop biomasses on soil surfaces under the anaerobic conditions in flooded rice systems produce organic acids (mainly acetic acid) that cause early phytotoxicity to rice seedling coleoptile and roots. This study evaluated the dose-response of acetic acid on germination rates and post-germination growth traits (coleoptile, first leaf, and roots). Under controlled conditions, the seeds of three rice varieties (Sant'Andrea, Salvo, and Selenio) were immersed in acetic acid concentrations (0, 9, 18, 36, 54, and 72 ppm) for eight days. Germination results suggest that acetic acid likely scarred var. Salvo, based on a 15% faster germination rate compared to untreated controls. Across all varieties, increased acetic acid concentrations never slowed germination. During post-germination growth stages, root phytotoxicity was always more evident than shoot phytotoxicity, although the responses varied among the varieties. Root length damage appeared first at acetic acid concentrations of 36 ppm in var. Sant'Andrea and Selenio, and at 54 ppm in var. Salvo. Root length measurements provided explicit and speedy information on varietal tolerance to acetic acid and, consequently to cover crop fermentation and suggested that direct observation of root damage in paddy fields is valuable for prompt water management decisions, such as flooding interruption. Further development of this method may lead to more complete varietal screening and identification of related genetic traits responsible for tolerance.

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Key words: Phytotoxicity; organic farming; genotypes; root; coleoptile; first leaf.

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Introduction

Italy, the largest producer of rice in Europe, yielded 1.6 million tons in 2017 (FAOSTAT, 2019), which represented more than half of Europe's total rice production (Masseroni *et al*., 2018). Italy is also the top European producer of organic rice (62,000 tons of grain annually) (EUROSTAT, 2019) and holds the largest percentage (7.6%) of the total European rice area (17,832 ha in 2018) (SINAB and FAOSTAT, 2019; EUROSTAT 2019). Italy's top spot in organic rice farming generally correlates with its organic farming growth, as Italy is also first in its share of total organic land (15.4% of agricultural land). On the global scale, Italy ranks as the fifth largest organic food market by value (3.1 billion euros) after the USA (40 billion euros), Germany (10 billion euros), France (7.9 billion euros), and China (7.6 billion euros) (FiBL, 2019).

Organic farming is often viewed as environmentally-friendly agriculture (Rigby and Càceres, 2001; Yadav *et al*., 2019). However, shortcomings have been illuminated over time, and the most significant one is that its techniques cause cash crop yields, in general, to fall by 20-30% (Niggli, 2014), winter wheat and maize to decline by 18.5% (Sacco *et al*., 2015), and rice to drop by between 6% (de Ponti *et al*., 2012) and 30% (Huang *et al*., 2016). Weed control (Towa *et al*., 2013) and nitrogen (N) availability (Huang *et al*., 2016) are the main factors limiting yield in organic rice farming. Both must be managed in a preventive way for the medium/long term, as chemical products are forbidden (Watson *et al*., 2002). Yield declines have also raised concerns that organic agriculture may pose future food security problems (Capone *et al*., 2014). Combined, these issues underscore one of the greatest challenges facing agriculture today, to create sustainable food chains that allow maintaining good yields adopting environmentally-friendly strategies (Reganold and Wachter, 2016).

One possible strategy is using cover crops to replace chemical N fertilisers and herbicides in organic agriculture (Campiglia *et al*., 2009; Hartwig, 2002; Haque, 2013). However, realising the benefits of reducing these chemicals simultaneously is not easy, and different management techniques typically optimise one at the expense of the other (Campiglia *et al*., 2010). Among the cover crop termination techniques available to control weeds effectively (Hartwig, 2002) by establishing a physical barrier that reduces weed germination and emergence is '*green mulching*'. The method lodges the crop by roller-crimping or shredding the remaining biomass and leaving it on the soil surface (Creamer and Dabney, 2002; Kornecki *et al*., 2009). The effects of cover crop use throughout the world are available in the literature for upland crops, but the effects of managing such crops under flooded field conditions are not fully known, and the few available studies sometimes report contradictory results. This is particularly important for rice as 75% of the world's supply is grown in flooded conditions (Peyron *et al*., 2016). Waterlogging has several agronomic advantages: it limits soil moisture, and temperature variations are limited; it depresses soil-borne diseases and upland weed growth; it avoids surface crust formation in heavy soils; and it prevents seed germination impairment (Lee *et al*., 1998). The most adopted rice management technique in Italy is waterlogging. This is particularly true in the Piedmont Region (northeastern Italy), where paddy field soils are mainly clayey and silty (Zampieri *et al*., 2019).

In flooded conditions, the combination of cover crops and water seeding produces peculiar effects. Indeed, rice paddy flooding induces increasingly anaerobic soil conditions and a consequential progression to anaerobic predominance in the active microbial population (Le Mer and Roger, 2001). Anaerobic bacteria degrade added organic matter through fermentation, which raises several organic acid concentrations (acetic, propionic, and butyric acids) (Takai, 1970; Le Mer and Roger, 2001). The most-produced organic acid is acetic acid (Lynch, 1978; Camargo *et al*., 2001), which reaches its maximum concentrations in soil pore water three to five days after a rice field begins flooding (Chandrasekaran and Yoshida, 1973; Bohnen *et al*., 2005). After peaking, acetic acid concentrations gradually decline due to direct volatilisation or microbial transformation to methane (methanogenesis) (Johnson *et al*., 2006). The release of acetic acid from rice straw fermentation has been observed in flooded rice paddies (Rao and Mikkelsen, 1977) and from the added biomass of cover crops (Camargo *et al*., 2001; Bohnen *et al*., 2005). The release rate of acetic acid depends on biomass quality (Camargo *et al*., 2001), soil type (Biswas *et al*., 2001), and soil management factors, including how to balance the chosen technique for cover crop termination with the scheduling of termination, flooding onset, and rice seeding.

The amount of acetic acid released into the soil pore water seems to depend on whether the biomass is incorporated into the soil or left on the soil surface. Cannell and Lynch (1984) found that acetic acid production is higher with green manure than with green mulching due to the lower concentration of oxygen - and higher anaerobic microorganism decomposition - within the soil as opposed to on the soil. According to Sousa *et al*. (2007), green manure produces more acetic acid at a sub-surface depth (0.05 m) than closer to the surface (0.025 m). Conversely, green mulching produces acetic acid concentrations as a function of the distance from the surface biomass in the opposite direction; more acetic acid is produced closer to the surface (0.025 m), and less is produced at shallower concentrations (0.05 m from the soil surface). Other studies report higher acetic acid concentrations with green mulching relative to green manure, attributing the differential to increased carbon oxidation that arises from distributing the organic matter in the biomass along the soil profile mechanically (Bohnen *et al*., 2005).

Acetic acid concentrations in flooding water have been studied less than in soil pore water. However, it is established that water-seeding rice cultivated after a cover crop can significantly affect seed germination, especially in cases of green mulching (Lynch, 1980). Specifically, water-seeded rice germinates on the soil surface where green mulching biomass ferments, while green manure organic acids diffuse into the soil, a process that decreases by 50% at a distance of just 0.015 m from the fermentation source (Cannell and Lynch, 1984). So, acetic acid phytotoxicity impacts germination with either technique only if the seeds and/or the germinated seedlings are close to the fermented residues.

Acetic acid is thought to accelerate seed germination by increasing membrane permeability and breaking seed dormancy to facilitate proton transport in the seed cells (Cohn *et al*., 1987), although some authors report other results (Neves and Moraes, 2005; Pacheco *et al*., 2007). The phytotoxic effects of acetic acid have also been observed during germination and the early phases after germination in various crops, such as barley (Lynch, 1978), maize and clover (Lynch, 1980), oats (Kopp *et al*., 2009), and wheat (Tunes *et al*., 2012). The effects observed in these crops include root, coleoptile, and leaf length reductions due to meristematic tissue damage (Lynch, 1978; Takijima, 1964; Kopp *et al*., 2009; Tunes *et al*., 2012). Similar phytotoxic responses to acetic acid appear in rice during its germination and post-germination phases (Biswas *et al*., 2001; Camargo *et al*., 2001; Rao and Mikkelsen, 1977). Roots are reported to be more sensitive than shoots to acetic acid (Takijima, 1964), particularly during the early phases after germination (Kopp *et al*., 2012). In fact, a few weeks after germination, the toxic effect on rice was also noted as an epidermal thickening of the lateral and hypodermal layers of the adventitious root apices (Armstrong and Armstrong, 2001). Problems in root growth can cause lower nutrient absorption (Fortes *et al*., 2008) and decreased yields. Prolonged exposure to acetic acid may also cause root death (especially at apices) and early senescence of the aerial part (Armstrong and Armstrong, 2001).

Different tolerances to acetic acid among rice varieties have been reported, notably at high concentrations (up to 12 mM, corresponding to 720 ppm) (Kopp *et al*., 2009; Biswas *et al*., 2001). However, few studies have investigated the effects of low concentrations of acetic acid on seed germination and early growth in different rice varieties. Such measurements could establish variety-specific acetic acid tolerance thresholds, which are less viable at high concentrations due to generalized injury in all varieties. It is expected that using a narrow range of acetic acid concentrations, it will be possible to identify the genotypes most tolerant to organic acids phytotoxicity, which could, in turn, facilitate the introduction of green mulching in paddy fields.

The objectives of this study were both methodological and agronomic. The main methodological objective was to identify a quick and effective method to correctly assess phytotoxicity in the early stages of germination based on the best indicators of growth stress. The primary agronomic objective was to analyse the germination performances of three varieties suitable for organic farming to identify preliminary indications of varietal tolerance, a study that should be extended to many genotypes. This information will be useful for the specific management of flooding water when green mulching is applied.

Materials and methods

Experimental setup

The experiment was conducted in a growth chamber under continuous light (PAR 160 µmol m^{-1} s^{-1}) and temperature control (20°C). The experimental units were glass jars (5 cm diam. × 10 cm height) containing germinating rice seeds placed on a shelf 1.50 m from the chamber floor. For each variety, ten seeds in each jar were immersed in acetic acid solutions of 0, 9, 18, 36, 54, and 72 ppm, with three replicates arranged in randomized temporal blocks. In total, there were 54 jars from a combination of six solutions, three rice plant varieties, and three replicates. All jars were filled until the acetic acid solution reached 6 cm from the bottom of the jar (118 mL of solution), following the procedure of Biswas *et al.* (2001). Controls (0 ppm acetic acid) were prepared with deionised water. To avoid solution evaporation, each jar was covered with a Parafilm® membrane.

The order of magnitude concentrations tested was selected based on a preliminary sampling campaign conducted by authors in paddy fields during green mulching biomass fermentation, which revealed acetic acid concentration peaks of approximately 30 ppm (unpublished data). Previous studies have considered higher concentrations based on acetic acid concentrations limited to soil pore water (Bohnen *et al.*, 2005; Johnson *et al.*, 2006; Kopp *et al.*, 2009). To our knowledge, no studies have evaluated various acetic acid concentrations in flooding water. As noted previously, this study deliberately defined a narrow range of acetic acid concentrations to discriminate varietal response.

The study tested three rice varieties: Sant'Andrea, Salvo, and Selenio. They are temperate *Japonica* rice varieties belonging to a different commercial type (Reg. EU 1308/2013), classified according to grain size and shape: Sant'Andrea is a long-grain A variety (length >6 mm, the length-to-width ratio between 2 and 3 mm); Salvo is a grain B variety (length >6 mm, length-to-width ratio >3 mm); Selenio is a round grain variety (length <5.2 mm, length-to-width ratio <2 mm. These varieties are found throughout Italy on both conventional and organic farms. All seeds were self-produced within experimental plots cultivated in the previous growing season under organic farming techniques and were immersed in deionised water for 24 h immediately before the experimental start to simulate the standard farm practice of pre-soaking rice seeds in water before water seeding.

The experiment duration was eight days after seed immersion in the glass jars. This period intended to simulate one of the most widespread water managements of continuous flooded cultivation in Italian paddies - *i.e.*, pinpoint flooding- that, as described by Miniotti *et al.* (2016) and by Peyron *et al.* (2016), provides an interruption of initial flooding after seeding, necessary for root anchoring. This implies that the contact of rice seeds with acetic acid occurs in a definite period (that, based on a local survey, is on average 8-day long) interrupted by drainage that both removes acetic acid itself and stops conditions for its production.

Germination response

To evaluate the dose-response effect of acetic acid on seed germination for the three selected varieties, the number of germinated seeds was counted daily from the beginning of acetic acid solution immersion until five days later. After that, germination was no longer observed. Each seed was deemed '*germinated*' once the tegument was torn by the growth of the apical tip (Bewley, 1997; Magneschi and Perata, 2009). To study the temporal dynamics of germination, data were analysed by applying a dose-response three-parameter log-logistic model adopted in previous similar studies (Ritz and Streibig, 2015, Lewandrowski *et al.*, 2017) using a time-to-event method approach (Onofri *et al.*, 2018) to obtain cumulative germination curves as a function of acetic acid concentration and variety.

Fitting was performed through the *drm* function available in the *drc* package in R (Ritz and Streibig, 2015), as shown in formula (1). The argument '*type*' was set to '*event*' in the function call.

$$f(x) = \frac{d}{1+\exp\left[b(\log(x)-\log(e))\right]} \qquad (1)$$

Where parameter '*d*', corresponding to the asymptote of the function, represents maximum germination defined as the ratio of germinated seeds over a hypothetical unlimited time; parameter '*e*' is the inflection point of the function and corresponds to the time when 50% maximum germination occurs ('*d*') is reached (t_{50}); parameter '*b*' is the slope of the function in correspondence of '*e*', which is related to the standard deviation of the log-logistic distribution.

Post-germination growth traits

Eight days after seed immersion, a destructive test was carried out to assess the effects of acetic acid on rice root and apical tip growth on some of the seeds in each jar (*i.e.*, 5 seeds per jar resulting in 15 germinated seeds per treatment). The timing of the measurement was determined considering that this period corresponded to the maximum time that commonly occurs between water seeding and the first drainage event in the typical management of rice paddies. Destructive measures included separating some roots, one coleoptile, and the first leaf from each seedling. Each component was then blotted on absorbent paper and placed on a dark-coloured plastic sheet to ensure proper black/white contrast. Colour images were acquired using the EPSON Perfection 4490 Photo scanner and were processed with Corel PhotoPaint Software to obtain 1-bit images, which were then analysed with KS-300 Image software rel. 3.0 (Zeiss, Carl Zeiss Vision GmbH, München, Germany) through an automatic procedure. The traits evaluated were length and diameter of roots and coleoptile and length and width of first leaves. In addition, the number of roots for each germinated seed was counted, and the total length was calculated as the sum of the lengths of all roots from a single seed.

Statistical analysis

For the germination response, to observe the differences between the logistics curves obtained for each variety at increasing concentrations, parameters *b, d,* and *e* were compared using the compParm function in the *drc* library. For growth traits, the effects of acetic acid were tested on the length and diameter of roots, coleoptile, first leaves, total root length, and the number of germinated roots by analysis of variance (ANOVA). Although a curve-fitting procedure applying the equation proposed by Brain and Cousens (1989) was initially considered for post-germination data analysis as indicated by Onofri *et al.* (2010), obtained results were not fully informative, and ANOVA associated with multiple comparison procedures was preferred. The fixed factors considered in the ANOVA model were: variety, concentration, variety × concentration, and block. After verifying the normality of the residues through the Shapiro-Wilk test and homoscedasticity through the Levene test, the *gls* model was used, which is included in the *nlme* package, which possibly admits the inhomogeneity of factor variances. Coleoptile properties (length and diameter) showed a normal distribution of residues and homogeneous variances, so the linear model *lm* was used. For diameters of roots and leaves, reciprocal transformation (1/n) of the measured values was necessary to meet the normality requirement.

The inhomogeneity of variances was modelled by assuming an independent structure for each variety and/or concentration, depending on how the variances had the better range for all the treatments tested. For total root length and mean root length, the variance structure has been considered independent for each concentration. In addition, for the first leaf length, the variance structure has been considered independent for each variety. When significant, treatment averages were separated through the Bonferroni post hoc test.

All analyses were performed with R Studio Software (version 3.4.3).

Results

Germination response

Cumulative germination curves were generated to reveal same-variety response differences associated with acetic acid concentrations (Figure 1). In general, the statistical analysis highlighted a significant effect on the parameter *e* (t_{50}) (Table 1). No tested variety demonstrated a clear proportional response to increasing acetic acid concentrations. In particular, germination accelerated in Salvo and Selenio with rising concentrations of acetic acid and reached its maximum speed (minimum t_{50}) at 54 ppm, but 72 ppm was an exception to the observed trend for both varieties while also 0 ppm for Selenio. Sant'Andrea, although having a maximum speed at 72 ppm, did not exhibit a trend with raised concentrations, showing the control (0 ppm) statistically equal at all acetic acid treatments.

Post-germination growth traits

Roots

The significant effect of the variety × acetic acid concentration interaction on total root length (RTL) and mean root length (RL) highlighted that the root response to acetic acid varied with variety (Figure 2). For Sant'Andrea, RTL shortening was not observed in

Table 1. Time necessary to reach 50% of seed germination (t_{50}) at different acetic acid concentrations and in different varieties ('*e*' of the dose-response three-parameter log-logistic function).

Concentration (ppm)	Sant'Andrea	Salvo	Selenio
0	1.57^{ab}	2.34^{c}	1.64^{abc}
9	1.92^{c}	2.17^{bc}	1.88^{c}
18	1.44^{ab}	2.10^{bc}	1.82^{bc}
36	1.50^{ab}	1.78^{ab}	1.45^{ab}
54	1.74^{bc}	1.73^{a}	1.41^{a}
72	1.34^{a}	2.03^{b}	1.68^{abc}

$^{a-c}$Letters indicate significant differences identified by *compParm* function.

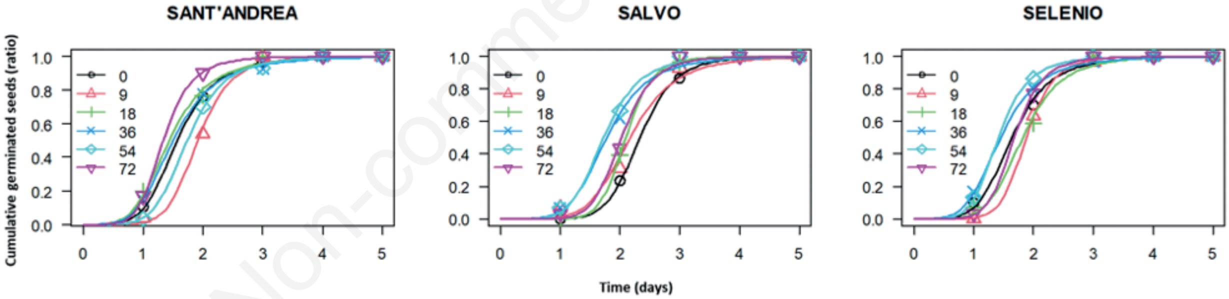

Figure 1. Cumulative germination curves. Cumulative germinated seed values (ratio) for days 0 to 5 after immersion in acetic acid solution. Each line presents the curve for each tested concentration (0, 9, 18, 36, 54, and 72 ppm).

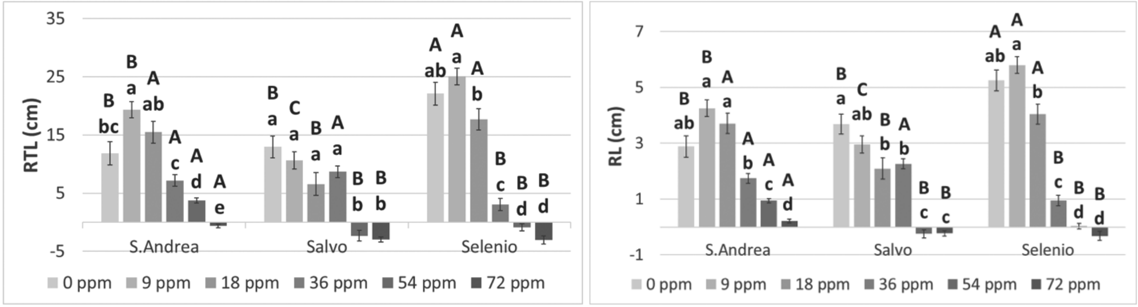

Figure 2. Root lengths in rice seeds during germination in various acetic acid solutions. Total root length (RTL) and mean root length (RL) for each variety at increasing acetic acid concentrations. Letters indicate significant differences identified with the Bonferroni *post-hoc* test (variety × concentration effect: P<0.000). Lowercase letters indicate differences in response to different concentrations of acetic acid within the same variety; uppercase letters indicate differences between varieties at the same acetic acid concentration. All values are estimated marginal means using ANOVA.

the less-concentrated treatments (0 to 18 ppm) but did appear at 36 ppm and progressively increased. Only in Sant'Andrea was RTL observed to be shortened early on (at 0 ppm) than at 9 ppm. In Salvo, the RTL reductions occurred at higher concentrations (54 and 72 ppm) *versus* the statistically equivalent 0, 9, 18, and 36 ppm treatments. Selenio RTL dropped sharply relative to the control, starting at concentrations of 36 ppm and progressively decreased.

At identical concentrations, RTL differed among the three varieties. At 0 ppm, Sant'Andrea equalled Salvo, while Selenio exceeded both. At 9 ppm, Selenio had the highest RTL value, Salvo the lowest, and Sant'Andrea was significantly different from the other two and intermediate. At 18 ppm, Sant'Andrea reached the RTL value of Selenio, and both were significantly higher than Salvo. At 36 ppm, the RTL of Selenio was the lowest, with a 61% decrease compared to the RTLs of Salvo and Sant'Andrea. At 54 and 72 ppm, Selenio maintained its lowest-ranking RTL, Salvo was similar to Selenio, and Sant'Andrea displayed the highest values.

The mean root length (RL) trended differently than RTL, except in the Selenio variety. Sant'Andrea showed no significant RL increase at 9 ppm relative to the control (0 ppm), contrary to that observed for RTL. Instead, for Sant'Andrea, a significant reduction in RL that began at 54 ppm had increased at 72 ppm. In the case of Salvo, the RL reduction observed first at 18 ppm fell significantly again at 54 ppm. When identical acetic acid concentrations were compared across the varieties, RL approximated the same differences as were observed for RTL.

Other changes in root measurements were observed following exposure to acetic acid. For example, the number of roots per seedling decreased, but only at high concentrations (Figure 3). Threshold concentrations were identifiable (statistically) for Sant'Andrea and Salvo at 72 and 54 ppm for Selenio. Additionally, the interaction of variety × acetic acid concentration resulted in significant changes to root diameters. In contrast to RTL values, solutions with higher concentrations of acetic acid caused increased root diameters (Table 2).

While Salvo showed no significant differences over the range of concentrations, a significant thickening was observed in Sant'Andrea and Selenio at 36 ppm when compared to the control. Furthermore, Selenio also displayed a significant increase in diameter at 54 ppm. Finally, upon comparing the varieties at the same concentrations, root diameters differed significantly at each concentration except at 72 ppm. Among root diameter results, it is noteworthy that Selenio exhibited the smallest diameters at nearly all concentrations, suggesting a genetic trait.

Coleoptile

Both variety and acetic acid concentration significantly affected coleoptile length; however, the interaction of the two variables was not significant. Coleoptile lengths decreased in all varieties (Figure 4) at the highest concentrations (54 ppm and 72 ppm). Significant differences in coleoptile lengths were also found across varieties, which ranked from highest to lowest as Sant'Andrea, Salvo, and Selenio. In terms of coleoptile diameters, only a varietal variation was noted; no significant influence of acetic acid concentration or the interaction of variety and concentration was detected. Coleoptile diameters were largest in Sant'Andrea (613 µm), intermediate in Salvo (503 µm), and smallest in Selenio (500 µm).

First leaf

Significant effects from both variety and acetic acid concentrations were made evident by the first leaf length (LL) analysis. No effect was found from their interaction. Leaf lengths were generally reduced at 72 ppm compared to relatively stable values between 9 and 36 ppm (Figure 5). The first LL illuminated a slight developmental difference by variety: Selenio (short), Salvo (intermediate), and Sant'Andrea (long). Last, first leaf width (LW) values were at

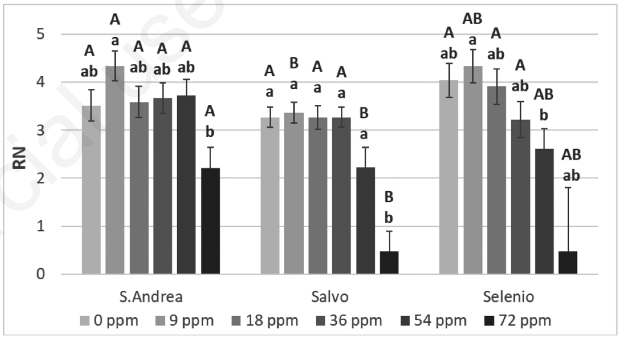

Figure 3. Number of roots in rice seeds during germination in various acetic acid solutions. Number of roots (RN) for each seed of three varieties at increasing acetic acid concentrations. Letters indicate significant differences identified with the Bonferroni *post-hoc* test (variety × concentration effect: P<0.000). Lowercase letters indicate differences in response to different concentrations of acetic acid within the same variety; uppercase letters indicate differences between varieties at the same acetic acid concentration. All values are estimated marginal means using ANOVA.

Table 2. Root diameter values.

Concentration (ppm)	Sant'Andrea		Salvo		Selenio	
	Reciprocal values (µm^{-1})	Mean values (µm)	Reciprocal values (µm^{-1})	Mean values (µm)	Reciprocal values (µm^{-1})	Mean values (µm)
0	0.0031	322.7$^{a\ B}$	0.0030	330.5^{B}	0.0041	248.6$^{a\ A}$
9	0.0029	343.7$^{ab\ C}$	0.0035	292.1^{B}	0.0043	233.6$^{a\ A}$
18	0.0030	335.5$^{ab\ B}$	0.0030	259.7^{B}	0.0038	266.3$^{a\ A}$
36	0.0027	373.6$^{b\ B}$	0.0033	272.8^{A}	0.0031	308.6$^{b\ AB}$
54	0.0028	373.0$^{ab\ B}$	0.0031	341.8^{AB}	0.0032	311.8$^{b\ A}$
72	0.0028	353.5$^{ab\ A}$	0.0028	348.6^{A}	0.0031	314.7$^{ab\ A}$
P(F) *Variety*			<0.001			
P (F) *Concentration*			<0.001			
P (F) *Variety × Concentration*			<0.001			

$^{A-C}$Letters indicate significant differences identified with the Bonferroni *post-hoc* test for variety × concentration interaction. $^{a-b}$Lowercase letters indicate differences in response to different concentrations of acetic acid; uppercase letters indicate differences among varieties within the same acetic acid concentration. All values are estimated marginal means using ANOVA.

their minimums at the highest concentration, with Salvo values consistently above those of the other two varieties (data not shown).

Discussion

Through tegument protein scarification, organic acids are known to accelerate seed germination. By increasing hull permeability, the transport of protons and water into the seed is facilitated, and dormancy factors are released. In addition, high proton concentrations are thought to reduce seed internal pH values, suggesting that coupled with membrane modification may permit germination inhibitors responsible for dormancy to exit the seed and accelerate the germination process (Cohn *et al*., 1987). However, during the second stage of germination, the effects of organic acids are manifested in reduced root and shoot elongation, with consequent loss in root biomass, nutrient uptake, and shoot development (Takijima, 1964; Biswas *et al*., 2001; Camargo *et al*., 2001). These negative effects have been observed in various cereals, including rice (Rao and Mikkelsen, 1977), barley (Lynch, 1978), oat (Ferreira da Silveira *et al*., 2014), and wheat (Tunes *et al*., 2012).

Both the increase of hull permeability and reduction in root and shoot growth are likely due to the effects of organic acids on cell membrane integrity/functionality and energy production (Camargo *et al*., 2001). As Tunes *et al*. (2012) describes, acetic acid can modify the selectivity of a cell membrane by altering its poly-unsaturated fatty acid content. Such changes tend to cause two concurrent effects: a decrease in the cellular content of proteins, sugars, and soluble starches and an increase in alpha-amylase activity that promotes starch hydrolysis into simple sugars and loss of energy reserves (Neves and Moraes, 2007). In flooded conditions, seed cell aerobic respiration is gradually reduced, then blocked, and cell division is inhibited (Camargo *et al*., 2001).

Phytotoxicity by acetic acid may vary widely among species and varieties. In particular, seed thickness and/or tegument coat composition can affect protons and water absorption and vary germination rates (Lee *et al*., 2003). For example, variable germination rate responses and root and shoot development outcomes linked to organic acid phytotoxicity have already been demonstrated in oats (Ferreira da Silveira *et al*., 2014). Similarly, the different effects of organic acids in flooding water have been shown in the phosphorus and potassium root absorption capacities of *Indica* and *Japonica* rice varieties in hydroponic systems (Kopp *et al*., 2012).

In this study, three *Japonica* rice varieties responded differently to acetic acid solutions in an artificial flooding system, for example, the time required for var. Selenio, to reach 50% maximum germination (t_{50}) in the acid treatments, failed to differ significantly from the control. Var. Sant'Andrea behaved similarly to Selenio, except for a germination slowdown at 9 ppm acetic acid solution as indicated by a 25% higher t_{50} value *versus* that of the

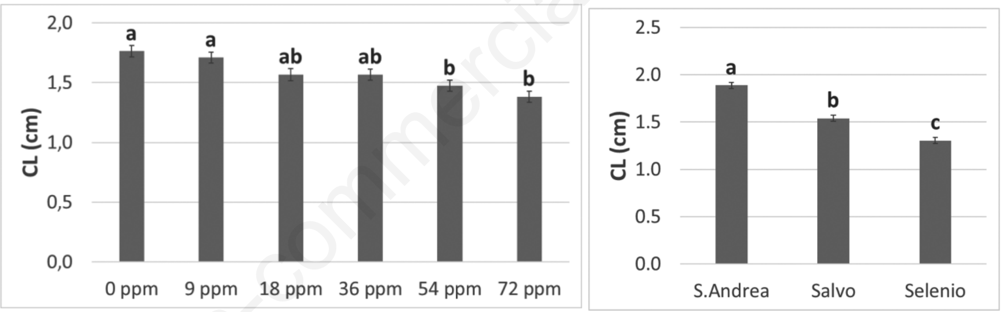

Figure 4. Coleoptile development in rice seeds during germination in various acetic acid solutions. Coleoptile average lengths (CL) of three varieties at each acetic acid concentration (left), and average lengths across all concentrations for each variety (right). Letters indicate significant differences identified with the Bonferroni *post-hoc* test (Concentration effect: P<0.000; Variety effect: P<0.000). All values are estimated marginal means using ANOVA.

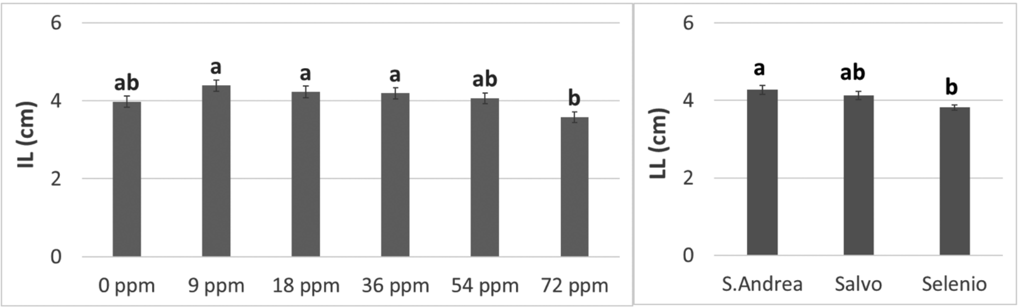

Figure 5. First leaf development in rice seeds during germination in various acetic acid solutions. First leaf average lengths (LL) of three varieties at each acetic acid concentration averaged for three varieties in each concentration (left), and average lengths across all concentrations for each variety (right). Letters indicate significant differences identified with the Bonferroni *post-hoc* test (concentration effect: P<0.000; variety effect: P<0.000). All values presented are estimated marginal means using ANOVA.

control. On the contrary, var. Salvo germination accelerated at acetic acid concentrations ≥36 ppm with respect to the control, which was demonstrated by 19% lower t_{50} values at 36 and 54 ppm than at the 0-18 ppm range. There was evidence that scarification likely occurred in Salvo at high acid concentrations as t_{50} fell 15% below the control values. The absence of similar changes in t_{50} values confirmed that progressive acceleration did not occur in the germinations of Sant'Andrea and Selenio. Cohn *et al*. (1987) showed acetic acid caused accelerated weedy rice seed germination, yet the comparison is elusive as the concentrations tested were much stronger in that study, up 53 mM (3000 ppm).

Among the three varieties considered in this study, none exhibited germination slowdown caused by a progressive increase in acetic acid concentration, which aligns with the results of other studies testing various organic acids (Neves and Moraes, 2005; Pacheco *et al*., 2007). Worthy of note in the current study is that observed differences in germination rates were generally modest. However, such changes may be amplified in the field depending on temperate climate temperatures at seeding during rice cultivation. The typically lower field temperatures compared with those used in this experiment would increase the time germination time and might amplify the varietal differences.

The post-germination effects of acetic acid observed in this study were consistent with previous studies (Rao and Mikkelsen, 1977; Biswas *et al*., 2001; Camargo *et al*., 2001). Both acetic acid concentrations and germination rates were shown to influence root and shoot development traits during this phase concurrently.

Regardless of variety, evidence of negative effects from acetic acid was more evident on root growth than shoot growth, which others have also reported (Rao and Mikkelsen, 1977; Biswas *et al*., 2001; Camargo *et al*., 2001). Not only have previous studies documented the high sensitivity of roots to organic acids (Armstrong and Armstrong 2001; Biswas *et al*., 2001; Camargo *et al*., 2001; Kopp *et al*., 2009), but also further research on the topic has postulated that root length reduction probably arises from direct contact of organic acids with meristems that consequently inhibits cell division in root apexes (Camargo *et al*.; 2001, Kopp *et al*., 2012).

In this study, roots were the only plant organ to produce a significant variety × acetic acid concentration interaction response. Consequently, observing root effects may offer information on the capacity of each variety to tolerate acetic acid. Unfortunately, despite the high sensitivity of roots to acetic acid, most studies have used much higher acetic acid concentrations than used here, making comparisons difficult. For example, Sousa and Bortolon (2002) found rice root length reductions of 50% at a very high (282 ppm) acetic acid concentration and a 50% shoot length reduction at 480 ppm after 14-day germination. Tavares *et al*. (2013) also reported 60% root and 19% shoot length reductions but at 720 ppm acetic acid concentrations.

Seeds germinated in flooded conditions induce fast elongation of the coleoptile and first leaf. This physiological response is described as the allocation of higher nutrient reserves from the endosperm to aerial parts of the plant rather than to the root system to overcome hypoxic or anoxic conditions (Ismail *et al*., 2009, Mondal *et al*., 2020). In the current study, this behaviour was observed in all the varieties tested, and no variety × acetic acid concentration interaction was evidenced for either coleoptile or first leaf development. However, what was noticed was that the first leaf appeared more tolerant than the coleoptile to acetic acid. This effect was attributed to the protection offered by the coleoptile itself that caused the first leaf to be in less contact with the acetic acid. Indeed, a reduction in the length of the first leaf was not evident before 72 ppm acetic acid, while significant coleoptile damage had already appeared at 54 ppm. Among the varieties, Sant'Andrea produced the longest and widest coleoptile and first leaf, while Selenio produced the shortest and thinnest.

The combined effect from both acetic acid concentration and elapsed contact time between roots and acetic acid solution (defined by germination rate) probably affected root damage. The Sant'Andrea and Selenio germination rates appeared to be unaffected by acetic acid concentration. However, because root exposure time was essentially constant across the different acid concentrations and the control, the root damage evident at 36 ppm can be considered the tolerance threshold for root damage in these two varieties. On the other hand, the accelerated germination that var. Salvo at 36 and 54 ppm resulted in a more prolonged exposure of the roots to acetic acid. While Salvo did not reveal root damage at 36 ppm, a sharp decrease in root length was exhibited at 54 ppm. It is purported that the slower germination rate of Salvo protected plant organs from the negative effect of the acid.

Variations in root diameter also highlight phytotoxicity by acetic acid on the root system. An increased root diameter can result from cell wall suberification and intercellular space lignification, which have been reported as mechanisms adopted by plants to prevent toxic substance entry (Armstrong and Armstrong, 2001). In varieties Sant'Andrea and Selenio, increases in root diameters generally trended with rising concentrations of acetic acid; however, var. Salvo showed no such significant effect in the short term. This phenomenon confirms the hypothesis that var. Salvo possesses a better intrinsic tolerance to acetic acid, although it does not preclude that longer contact with acid solutions might induce a similar response.

Conclusions

The low and tight range of acetic acid concentrations used as a methodology to test rice seed and seedling acetic acid phytotoxicity under flooding water was demonstrated to be suitable for screening the behaviour of the tested genotypes and potentially of a broad set of varieties. In addition, this procedure and the eight-day duration of seed immersion appropriately highlighted germination rate and growth trait differences during early seedling development.

Germination rate did not indicate acetic acid phytotoxicity because the effect of variety was stronger; in fact, two of the three varieties tested showed no acceleration or deceleration of the process. Root development traits were most sensitive to acetic acid phytotoxicity in the very early phase of germination. Therefore, root development traits could potentially provide the quickest and most effective indication of a variety of responses. Moreover, observing root damage in the field can allow timely interruptions of flooding so that the consequent fermentation of biomass from green mulching can be mitigated.

It would seem that the most suitable varieties to be cultivated in combination with the green mulching technique in water seeded rice are those with slow germination rates, protecting roots and shoots from direct contact with the acids. This study demonstrated that Salvo had slow intrinsic germination that apparently raised its tolerance threshold. Varieties that possess this behaviour are likely more able to tolerate prolonged periods of biomass fermentation on the soil surface. On the contrary, varieties having responses similar to that of Selenio and Sant'Andrea (fast intrinsic germination) can exploit short periods of the initial flooding to attain optimal concentrations of acetic acid formation to control weeds without compromising rice development. In the future, screening can be extended to a range of varieties, making it possible to identify the genetic traits responsible for the best adaptation to this agronomic technique.

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