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ABSTRACT

Ethanol is known to accumulate in various plant organs under various environmental conditions. However, there are very scarce data about ethanol sensing by plants. We observed that ethanol accumulates up to 3.5 mM during tomato seed imbibition, particularly when seeds were stacked. Stacked seeds germinated less than spread out seeds suggesting ethanol inhibits germination. In support of this, exogenous ethanol at physiological concentrations, ranging from 1 to 10 mM, inhibited germination of wild type tomato seeds. However, the germination pattern over the whole ethanol concentration range tested was modified in an ethylene insensitive mutant, *never-ripe (nr)*. The effects of exogenous ethanol were not linked to differences in ethylene production by imbibed seeds. But, we observed that exogenous ethanol at a concentration as low as 0.01 mM down regulated the expression of some ethylene receptors. Moreover, the triple response induced by ethylene in tomato seedlings was partially alleviated by 1 mM ethanol. Similar observations were made on Arabidopsis seeds. These results show there are interactions between ethylene sensing and ethanol in plants.

1. Introduction

Ethanol is a natural product accumulating in plant organs particularly when exposed to anaerobic conditions [1,2], to various other stresses such as seed deterioration [3], or fungus attack on the roots [4] and during ripening of fruit tissues [5]. Whether these ethanol levels are perceived by plant organs, and how, still remains an open question.

Levels at which ethanol accumulates in plant organs vary according to organs and plant species, and is often expressed in various units. In the following text, to make comparisons easier, we converted them all to mM. Cossins and Turner (1963) observed that germinating pea seeds accumulated ethanol to approximately 10 mM [6]. During the imbibition phase, soybean seeds accumulated ethanol to approximately 1 mM [3]. Accumulation of ethanol under anaerobic conditions has been observed ranging from 15 to 150 mM in cottonwood roots and leaves [1] and 0.2 2 mM in rice roots and leaves [2]. In fungal infected pine tree roots, Kelsey et al. (2016) found ethanol concentrations increasing to approximately 1 mM [4]. In pear fruit, ethanol is also produced at 1 to 20 mM while ripening [5]. Thus, ethanol accumulates into the mM range in a variety of organs and under a variety of conditions.

Research regarding ethanol perception by plants, includes the following studies. Miyoshi and Sato (1997) found that 400 mM ethanol stimulated rice germination, whereas, 100 mM slightly inhibited it [7]. Related to this, 200 mM ethanol treatment induces several genes in rice panicles [8]. Other effects of ethanol include induction of potato tuber sprouting by 100 mM ethanol [9] and induction of several genes by 500 mM ethanol in sugarcane [10]. Drawbacks of these studies are that ethanol concentrations *in planta* were not measured and the levels of ethanol tested were often higher than what has been reported to occur in plants.

More recently, Nguyen et al. (2017) found that 50 mM ethanol enhances high salinity stress tolerance in Arabidopsis and rice seedlings [11]. Because inhibition of Arabidopsis germination by NaCl is modulated by ethylene receptors [12], we decided to test whether or not ethanol affects ethylene perception. We initiated the study by working with tomato seeds, commonly used in our laboratory. We checked ethanol accumulation in seeds upon imbibition, and then tested the effects that physiological levels of ethanol have on germination of wild type and an ethylene insensitive mutant. These results on seed germination were compared to the effects that ethanol has on the transcript

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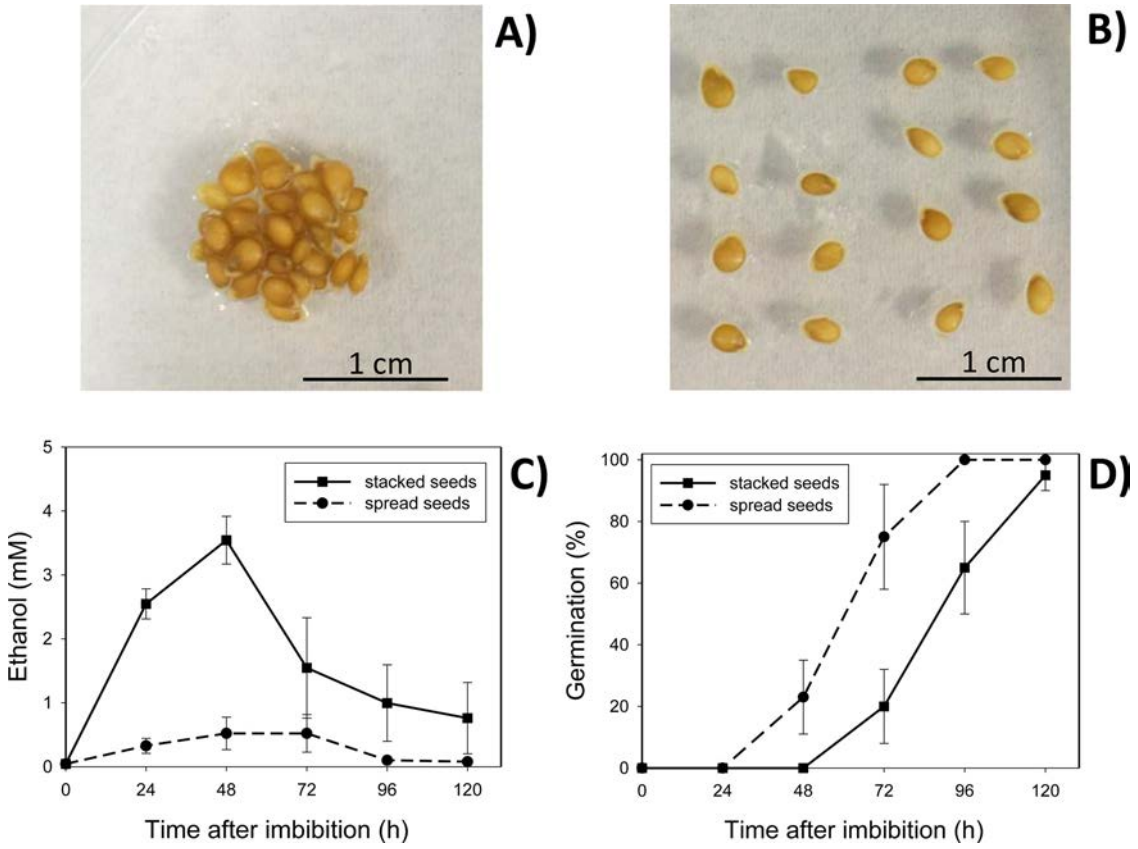

Fig. 1. Photographs to show distribution of A) 40 seeds stacked in a 1 cm^2 area and B) part of the 40 seeds spread over a 10 cm^2 area; C) ethanol content in germinating seeds that were either stacked or spread out, over time after imbibition; D) germination time courses of seeds that were either stacked or spread out; in panels C and D, n = 3 batches of 40 seeds, error bars show SE.

levels of several ethylene sensing and responsive genes. Finally, we checked the impact of ethanol on the triple response induced by ethylene. We then confirmed some of these observations using Arabidopsis seeds and seedlings. Our results show that low concentrations of ethanol affect plant germination and responses to ethylene and that ethylene signal transduction impacts these ethanol responses.

2. Materials and methods

2.1. Plant material

Tomato (*Solanum lycopersicum*, cv. Micro tom) seeds were sterilized with 5% NaClO for 10 min and washed with sterilized water for 3 4 times. Unless otherwise specified, the seeds were then spread approximately 0.8 cm from each other onto a Petri dish (Fig. 1D) containing 1/2 strength Murashige and Skoog salt mixture and germinated in a dark room at 22 °C. Two tomato lines were used: wild type and *never ripe* (*nr*) which is a gain of function mutant of the etr3 ethylene receptor. Arabidopsis (*Arabidopsis thaliana*, cv. Columbia) seeds were sterilized and grown on similar medium as the tomato seeds. Arabidopsis seeds were stratified for 48 h at 4 °C, before being transferred to light for three hours, then in the dark growth chamber as described above. Two Arabidopsis lines were used: wild type Col 0 and *etr1 1* which is a gain of function mutant of the etr1 ethylene receptor.

2.2. Ethanol measurements

Germinating seeds were frozen in liquid nitrogen and ground in a mortar and pestle. The powder was thawed in an Eppendorf tube while centrifuging at 16,000 *g* for 3 min at 4 °C. Ethanol content was then immediately assayed on the supernatant using an enzymatic kit (Biosentec, France) and spectrophometric measurements at 340 nm.

2.3. Exogenous ethanol treatment

Spread out seeds were exposed to various ethanol concentrations continuously while germinating. Ethanol was incorporated in the agar at 40 °C just before pouring it into Petri dishes. Final ethanol concentrations in the solidified agar were measured using the enzymatic kit, as described above.

2.4. Ethylene measurements

Twenty germinating tomato seeds were transferred to 2 ml vials at various times after the start of imbibition and incubated for 4 h at 22 °C. Headspace samples were analyzed with a gas chromatograph using a 2 m x3 mm 80/100 alumina column, an injector at 110 °C, N_2 as vector gas in an isocratic oven temperature at 70 °C, and a FID detector at 250 °C, as described before [13]. Preliminary experiments showed the maximum ethylene production was reached at 72 h after imbibition in our conditions.

2.5. qPCR analyses

Seeds were frozen with liquid nitrogen after 72 h imbibition under various conditions (control, ethanol, different tomato lines). Samples were ground to a frozen powder using mortar and pestle with liquid nitrogen. 50 mg of frozen sample was used for extracting RNA with a Promega RNA kit. The total RNA sample was treated with DNAseI (Ambion) to remove DNA. 1 µg RNA was used for reverse transcription with the Promega RT protocol. qPCR was performed as described previously [14]. All qPCR primers are listed in Supplementary Table S1.

2.6. Statistical analyses

The number of replicates is shown in each figure legend. The one way ANOVAs and multiple comparison tests were performed with Sigmaplot v11.0 (Systat Software Inc.).

3. Results

3.1. Stacking seeds increased ethanol content and delayed germination

We first confirmed that ethanol accumulates in germinating tomato seeds (Fig. 1) within the range of concentrations observed in germi nating pea seeds [6]. Unexpectedly, we observed differences depending on whether the seeds were spaced closely (Fig. 1A) or farther apart (Fig. 1B). When seeds were stacked in a very small area, they accu mulated more ethanol, reaching 3.5 mM (Fig. 1C), than when seeds were spread out over a larger area where the ethanol content reached only 0.5 mM. Additionally, the stacked seeds had a longer delay in germination onset than seeds spread out over a wider area (Fig. 1D).

3.2. Exogenous ethanol delayed germination, but alteration of ethylene perception modulated this trait

Because of prior links we have observed between ethylene signaling and ethanol [12], we tested exogenous ethanol effects on wild type and ethylene insensitive tomato seeds. For this and the remaining experi ments of this article, seeds were spread out to minimize accumulation of endogenous ethanol. We used a wide range of ethanol concentra tions. But even the highest concentration used (10 mM) is a low con centration being only 0.058 % (v/v) (Supplementary Table S2).

In wild type seeds (Fig. 2A), a statistically significant reduction in germination rate was observed at doses above 1 mM and possibly at lower levels (0.01 to 1 mM), but the latter responses were not statisti cally different from untreated seeds ($P > 0.05$). To determine if these ethanol concentration ranges affected other plant species, we examined Arabidopsis seeds and observed similar results where all tested ethanol doses (from 0.01 to 1 mM) inhibited seed germination (Fig. S1A) compared to the untreated controls. Thus, Arabidopsis seeds, which are smaller than tomato seeds, seem more sensitive to ethanol than tomato seeds.

By contrast, the ethylene insensitive *nr* mutant tomato seeds (Fig. 2B) show a different pattern. In the absence of ethanol, *nr* seed germination is lower than wild type seeds. Arabidopsis ethylene in sensitive *etr1 1* seeds also have reduced germination compared to wild type in the absence of ethanol (Fig. S1B). Interestingly, lower doses of ethanol that fail to affect germination of wild type tomato seeds, sti mulate germination of *nr* seeds (Fig. 2B). At higher dosages above 1 mM, *nr* seed germination is inhibited much like what was observed with wild type seeds.

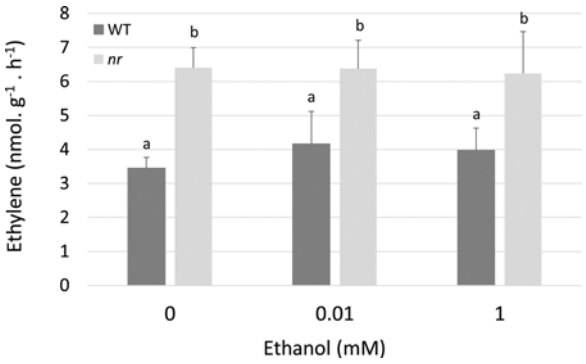

Fig. 3. Effects of exogenous ethanol on ethylene production by tomato seeds, 72 h after imbibition, wild type (WT) and Never Ripe mutant (*nr* is an etr3 gain-of-function mutant). n = 4 batches of 20 seeds, error bars show SE; small letters show differences by Tukey's HSD multiple comparison ($P < 0.05$).

3.3. The effects of ethanol on seed germination are not caused by changes in ethylene production

The above results indicate that ethylene signaling affects responses to ethanol in germinating seeds. To test if ethanol was inducing var iations in ethylene biosynthesis, we measured ethylene production of germinating seeds 72 h after imbibition. As shown in Fig. 3, in the absence of exognous ethanol germinating *nr* seeds produced more ethylene than wild type tomato seeds. Exogenous ethanol did not alter ethylene production by either seed line.

3.4. Ethanol modulates the expression of genes involved in ethylene sensing

To check whether exogenous ethanol was altering ethylene sensi tivity in the early stages of tomato seed germination, we analyzed the transcript levels of several genes that encode for proteins involved in ethylene signaling, or for known ethylene responsive genes (Fig. 4). Out of the 7 ethylene receptors (ETRs), we observed *ETR2* and *ETR4* were significantly affected by ethanol. A consistent decrease in *ETR7* ex pression was also observed, but it fell below the statistical cutoff ($P < 0.05$). *ETR4* expression was significantly down regulated by 0.01 mM ethanol, a dose that is 300 fold lower than the ethanol content

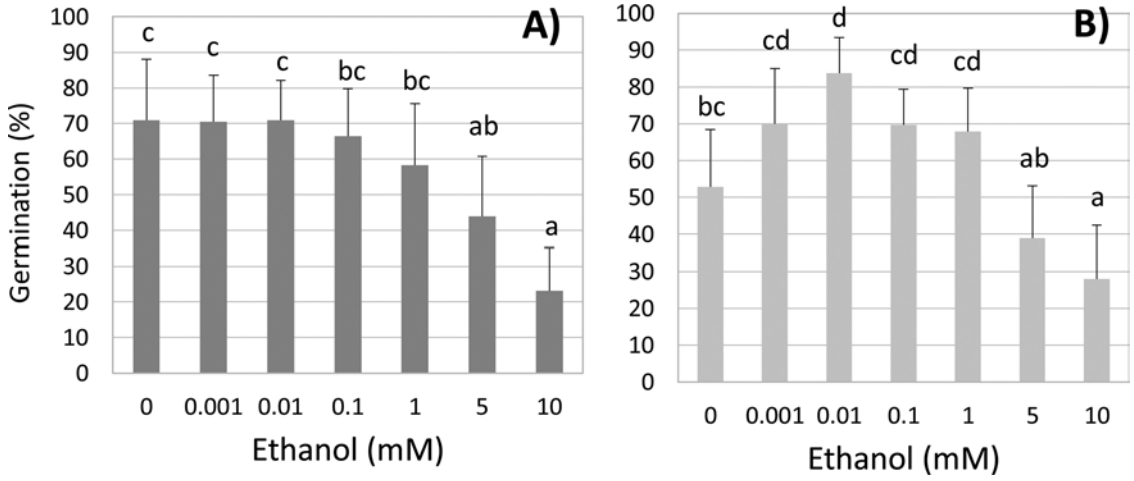

Fig. 2. Effects of exogenous ethanol on 'spread out' tomato seed germination at 72 h. A) wild type; B) Never Ripe mutant. For both panels, n = 10 batches of 10 seeds, error bars show SE; small letters show differences by Tukey's HSD multiple comparison ($P < 0.05$).

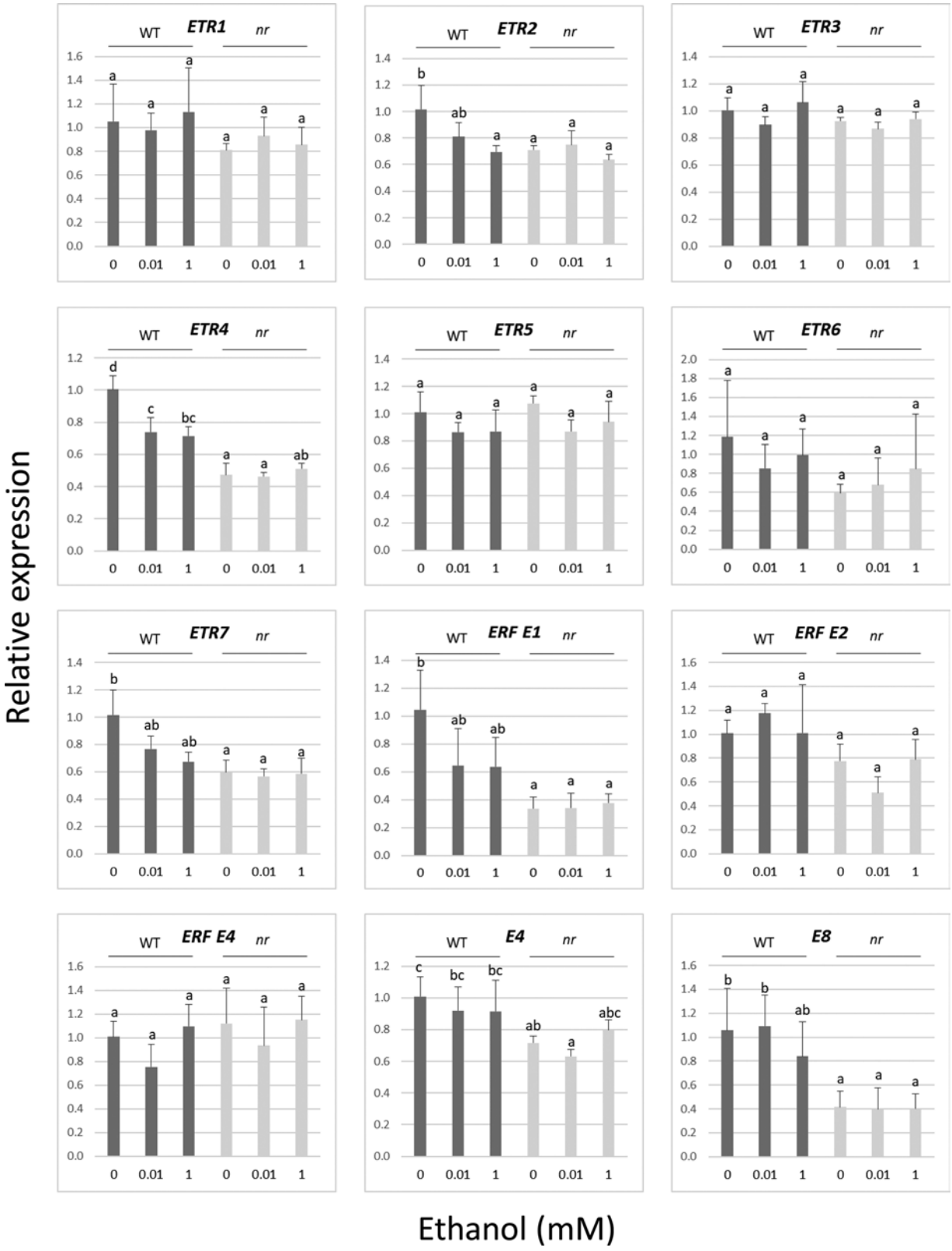

Fig. 4. Effects of exogenous ethanol on expression of genes that encode proteins involved in ethylene sensing and ethylene responsive genes. RNA was isolated from tomato seeds (wild type (WT) and Never Ripe mutant (*nr*)) 72 h after imbibition. Relative expression to WT at 0 mM ethanol is shown for each gene. n = 3 different seed batches, error bars show SE; different letters indicate statistical differences by Tukey's HSD multiple comparison ($P < 0.05$).

observed when stacking seeds for germination (Fig. 1A). *ETR2* was also down regulated at 0.01 mM, but the reduction only became significant at 1 mM ethanol. *ETR2* is the most highly expressed ethylene receptor in germinating tomato seeds (Fig. S2).

We also examined the transcript levels of several other genes involved in the ethylene signaling pathway. In wild type seeds, none of these genes were significantly affected by application of ethanol. However, *ERF E1* levels decreased with application of ethanol, but this

change fell below the statistical cutoff ($P < 0.05$). The abundance of *ERF E1*, but not the other *ERF*s observed, was lower in *nr* compared to wild type, as has been observed previously in tomato fruit tissues [15].

We also examined the expression levels of two genes, *E4* and *E8*, that have long been known as typical "ethylene responsive" genes [16,17]. Neither gene was down regulated by ethanol, but both were down regulated in the *nr* mutant compared to wild type. This is consistent with the idea that ethylene is not sensed by *nr* seeds, leading to down regulation of ethylene responsive genes such as *E4* and *E8*.

3.5. Ethanol reduces the hypocotyl shortening induced by ethylene

To further explore the links between ethanol and ethylene, we examined ethylene responses in dark grown seedlings. The *nr* gain of function mutation is insensitive to ethylene making it unable to show the "triple response" when ethylene is applied. This is a hallmark ethylene response in dark grown eudicot seedlings. In Arabidopsis and tomato, it includes a shortening of the root and hypocotyl, a thickening of the hypocotyl, and an exaggerated apical hook [18,19].

Thus, we tested whether or not ethanol impacts the growth of dark grown seedlings in either air or when ethylene is applied. As expected, application of 1 ppm of ethylene reduced the height of the tomato seedlings (Fig. 5). This effect was partially alleviated by concomitant exposure to 1 mM ethanol. A similar effect of ethanol was obtained with dark grown Arabidopsis seedlings (Fig. S3). Together, our results suggest that one effect of ethanol is to partially interfere with ethylene perception.

4. Discussion

We observed that ethanol content increased in tomato seeds after imbibition. There was higher ethanol accumulation in stacked seeds than in spread out seeds, and this higher accumulation correlated with slower germination. We have previously shown that high levels of ethanol reduce seed germination in Arabidopsis [20]. In the current study, we extended this to show very low levels of ethanol also impact the rate of seed germination in both tomato and Arabidopsis. Results by Nguyen et al. (2017) and Wilson et al. (2014) suggest ethanol and ethylene may be interacting to regulate seed germination [11,12]. Our observation that ethylene insensitive mutants such as *nr* in tomato and *etr1 1* in Arabidopsis have altered responses to ethanol during seed germination supports this idea. Additionally, ethanol affected ethylene responses in dark growth tomato and Arabidopsis seedlings suggesting that there is a reciprocal relationship between ethanol and ethylene.

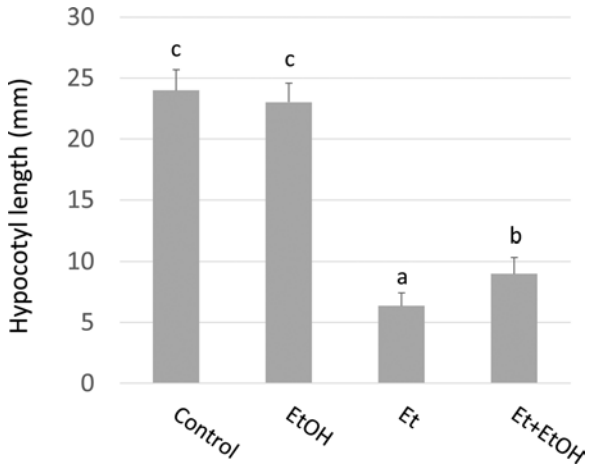

Fig. 5. Hypocotyl length of WT tomato seedlings; exposed to 1 ppm ethylene (Et) and/or 1 mM ethanol (EtOH), measured after 6 days of growth in the dark; n = 16 individual seedlings, error bars show SE, small letters show statistical differences by Tukey's HSD multiple comparison ($P < 0.05$).

Since ethylene production increases during seed germination and plays an important role in controling germination [21], we wished to determine whether or not ethanol levels affect ethylene production by germinating tomato seeds. We observed that exogenous ethanol does not change ethylene production by germinating wild type or *nr* seeds. However, the *nr* ethylene insensitive mutants produced more ethylene than wild type. A similar increase in ethylene production has been observed in *etr1 1* Arabidopsis mutant plants [22] and transgenic melons and petunia flowers expressing the Arabidopsis *etr1 1* gene [23,24]. Together, these results indicate that ethylene sensing, rather than biosynthesis, affects ethanol responses in germinating seeds.

Exogenous ethanol down regulated *ETR2* and *ETR4*. *ETR2* is the most expressed ETR in germinating seeds. Since mRNA content of the ETRs is positively correlated to ETR protein levels in other tomato tissues [25], this suggests ETR2 may have a larger role than the other receptor isoforms in regulating germination. ETR7 was also down regulated by ethanol. These observations indicate that ethanol is altering the ethylene sensing capacity of germinating seeds by down regulating the expression of some *ETR* isoforms.

Our results suggest that ethanol accumulation in tomato seeds delays germination; an effect partly linked with alterations in ethylene sensing. Since eliminating ethylene perception did not eliminate responses to ethanol, there are likely other pathways by which plants perceive these low ethanol concentrations.

5. Conclusions

Our observations support a model in which plants are able to sense ethanol through alterations in ethylene signal transduction. The fact that many plant organs accumulate ethanol under various stress conditions points to the importance of future studies determining the mechanism for ethanol perception in diverse plant tissues.

It is important to note the levels of ethanol used in this study are similar to, or lower than, ethanol levels often used to dissolve various chemicals such as auxins, gibberellins or brassinosteroids and care should be taken with proper controls, as mentioned previously [26]. For instance, 0.01 mM ethanol that led to altered ETR4 expression in tomato seeds corresponds to 0.00006 % (v/v) (Supplementary Table S2), showing that plants can respond to very low levels of ethanol.

Author contributions

YC, GD, and CC conceived and designed the experiments; YC, RAA, ED and EC carried out the experiments; YC, GD, BMB and CC participated in the data analysis; CC and BMB wrote the manuscript; YC and GD helped to review and edit the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2019.110368.

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