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Ethylene and Chitosan Affected the Seed Yield Components of Onion Depending More on the Dose Than Timing of Application

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Abstract: (1) Background: the production of onion seeds is limited by the competition between seeds and the vegetative organs and by scape lodging. However, information on the effects of plant growth regulation on onion seed production is scarce. Aim of the present study was to evaluate the seed yield components and germination ability of onion seeds as affected by the timing and dose of an ethylene application, a plant growth regulator able to modulate shoot–flower competition; and chitosan, an elicitor of plant defense mechanisms able to increase its tolerance to various stresses. (2) Methods: Onion was treated with ethylene at the recommended dose (100% RD) of a commercial product, at 150% RD in two contrasting phenological phases or untreated (control), or 'with' or 'without' chitosan, and the seed yield components and germination trend were measured. (3) Results: 100% RD at an early phase of growth did not influence the seed yield and increased the thousand seed weight (TSW) by 3.2%. The application of 150% RD decreased the seed yield by 33.5%, and this occurred irrespective of the timing of application. Such decreases were due to a reduction in the number of seeds per flower. The application of chitosan did not affect the crop at 100% RD and increased the seed yield and slightly increased, but not significantly, the TSW under 150% RD. Germination of the fresh seed was 92%, and 17 months of aging reduced it by 14%, with no effects of the treatments on the germination pattern. (4) Conclusions: the ethylene application mostly affected TSW but not the yield, whereas high doses of ethylene reduced yields irrespective of the timing of application. Such a result may have been due to a delay in the flowering onset that occurred in a relatively dry month. Chitosan sustained its yield when the yield potential was reduced by 150% RD, and such a result was likely due to physical protection from the transpiration since the synthetic fungicides applied did not likely allow the pathogens to infections. These results have implications for establishing the timing and dose of application of plant growth regulators and elicitors in seed onions to sustain the seed quality.

Keywords: *Allium cepa*; biostimulant; Mediterranean; plant growth regulators

Citation: Vecchiotti, D.; Angeletti, F.G.S.; Romanazzi, G.; Mariotti, M.; Saia, S. Ethylene and Chitosan Affected the Seed Yield Components of Onion Depending More on the Dose Than Timing of Application. *Horticulturae* **2022**, *8*, 781. https://doi.org/10.3390/horticulturae8090781

Academic Editor: Sergio Ruffo Roberto

Received: 18 July 2022
Accepted: 23 August 2022
Published: 28 August 2022

1. Introduction

Onion (*Allium cepa* L., Amaryllidaceae) is an important vegetable crop, representing 9.1% at a global scale of the total vegetable crops (FAO/STAT data [1], categories "Onion, dry" and "Vegetables Primary"). The onion growing area is increasing, on average, by 5.94% yearly from 1961 to 2020, with a sharp increase from 1991 onward. These trends confirm the strategic importance of the species for both agriculture and food security, and industrial uses [2]. Onion is a cold season, biennial crop bearing an edible bulb, whose propagation usually occurs by seed to better support the strong increase in the growing area, especially in small-farmer agriculture, despite other vegetative strategies being available [3–6]. The harvest of the crop for bulb production eliminates the chances of producing the seed, so

that dedicated crop from the bulb is used for the seed production. However, the optimal agronomic conditions for bulb production strongly differ from those for seed production [7]. Such as bulb production crops, seed production crops of onion rely on a range of conditions, including the genetic status of the crop, the availability of nutrients, the plant spacing, and agronomic management [7–10]. However, when aiming at seed production, competition between the vegetative part, especially the bulb, and the flowers may affect the seed yield and require specific management strategies to increase the seed yield and quality [5,11,12], which is a prerequisite for a good-standing establishment [13]. In particular, high resource availability and crop vigour, frequently due to its genetic traits [14], may strongly compete with seed production while, at the same time, increasing the chances of crop lodging and further pathogenic and pest attacks or mechanical yield losses, and seed quality reduction [15,16].

Thus, management practices to improve the seed yield should include increasing nutrient and water availability and improving the plant setting while at the same time avoiding stimulating the vegetative growth of the species [17–19].

Application of Plant Growth Regulators (PGRs) and biostimulants have been frequently tested in vegetable crop production to regulate a number of processes, including water availability, tolerance to stress, transfer of resources to the seed, and the vegetative propagation efficiency [20–25]. In particular, various PGRs may have contrasting effects on the onion physiology, and these effects depend on the degree of stresses that the crop may experience, the crop genotype, and the amount and timing of application, with some PGRs either improving or depressing given physiological traits depending on the rate of application [26]. Ethylene is a plant hormone able to strongly affect the bulb plant physiology [27]. Ethylene stimulates senescence and can strongly reduce the root, bulb, and shoot growth while increasing the senescence of all the plant organs depending on the concentration and stimulation of the transpiration, including under limited water conditions [28–30]. Nonetheless, the effect of ethylene on onion growth was seen to be either increased or in competition with other endogenous or exogenous PGRs [31]. However, the roles of ethylene on the yield components of the seed production of onion are scarcely known [15,32], as well as information on the effects of the ethylene dose. Chitosan is a natural biopolymer with antimicrobial effects and eliciting and film-forming properties that are widely used in the management of pre- and postharvest diseases and other stresses in plants, including its ability to reduce the transpiration and thus sustain the crop under arid conditions irrespective of the presence of biotic stresses [33]; despite these effects, they might strongly depend on the plant genotype, as seen in Tomatoes, and environmental conditions [34]. Since it is used in human medicine, and because it does not harm humans or the environment, chitosan was approved as a basic substance and it can be useful in replacing synthetic pesticides [35].

The aim of the present study was thus to evaluate the role of the dose and timing of the application of ethylene on the yield components of the onion for seed production in Mediterranean field conditions. In addition, a high dose of ethylene application in two phenological phases (either mid or late applications) was coupled with either the application of chitosan or not as a leaf coating to reduce transpiration and thus evaluate the interaction between high doses of ethylene application, their timing of application, and the eliciting effects.

2. Materials and Methods

2.1. Study Area, Site Description, and Agronomic History of the Experimental Field

The field experiment was conducted at the Daniele Vecchiotti farm (Magliano di Tenna, FM, Marche region, Italy),. The area of study has a south-eastern aspect, with a slope of about 2%, and is located about 250 m above sea level. The soil of the experiment is a silty loam with pH = 7.55; soil organic matter (Walkey Black) = 12.29 g kg^{-1}; Olsen-P = 39.3 ppm; and a coarse fraction <1%. At the time of planting, the soil had a total N = 1.40 g kg^{-1}.

The area has a warm Mediterranean climate with short dry periods in the summer (Figure 1, rainfall data of the Montegiorgio weather station, provided by ASSAM- Centro operativo di agrometereologia, Agenzia Servizi al Settore Agroalimentare delle Marche, period 1 January 2000–31 December 2019). The growing season was characterized by a rainfall (641.4 mm from October to July) lower than the long-term average (714.3 mm from October to July), with a dry winter, during which the rainfall was 32.8 mm and 57.9 mm lower than the long term, respectively.

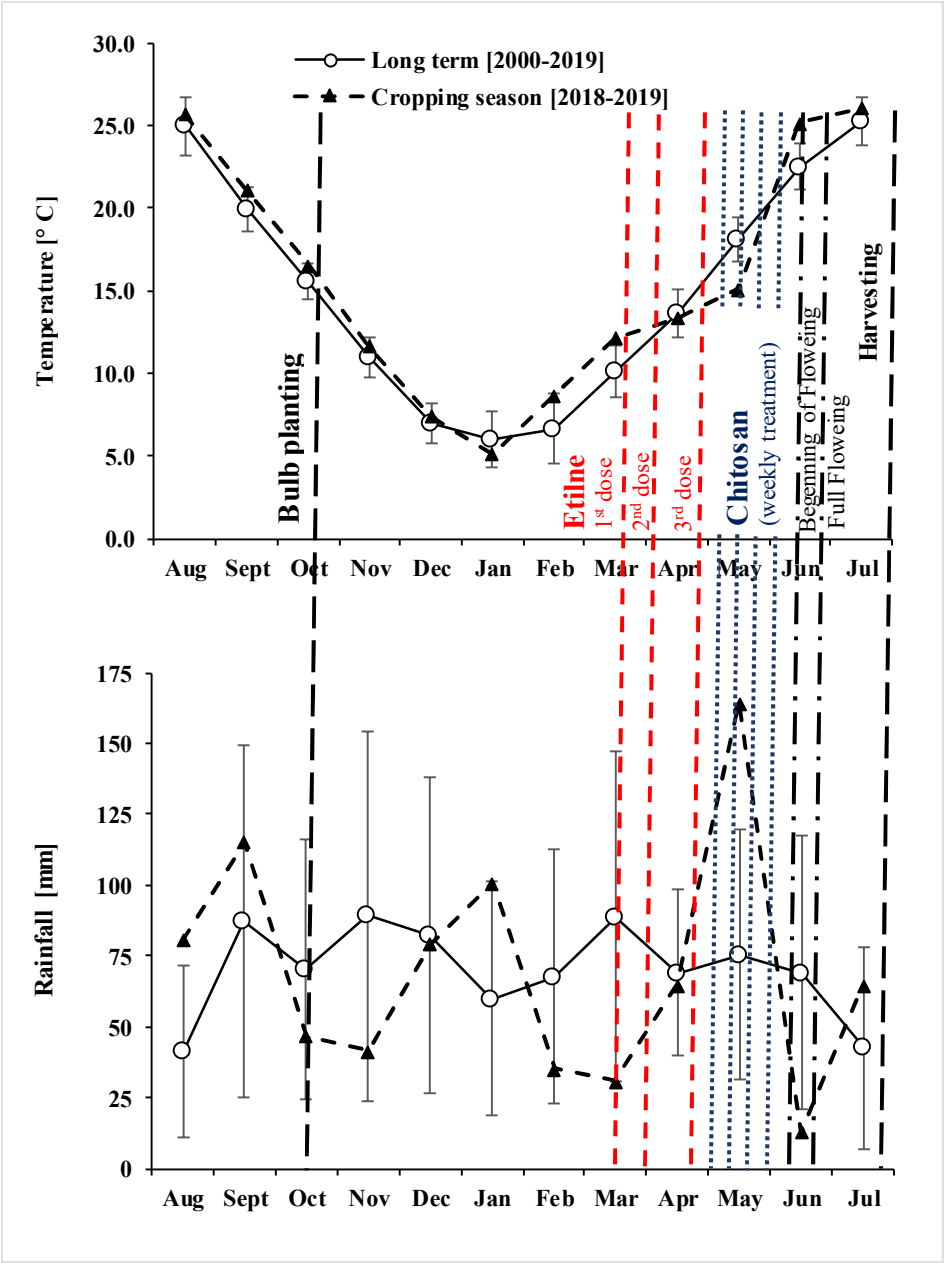

Figure 1. Long term (2000–2019) and cropping monthly season temperatures close to the experimental site. The figures report the dates of the onion bulb planting and infructescence harvesting (black long-dashed lines), the dates of the 3 moments of Ethylene application (red short-dashed lines), the dated of the chitosan application (blue dotted lines) and the dates of the beginning and full flowering (black dash-dotted lines). Moment of ethylene application included an early, mid, and late applications indicated as first, second and third (1st, 2nd, and 3rd, respectively) occasions in the figures. Amount of ethylene application included 100% recommended rate (in the 1st or 3rd occasions) or 100% in the 1st + 50% in either the 2nd or 3rd occasions. Chitosan application was performed in "Treat" including

a 100% application in the 1st occasion (irrespective of the further 50% application in the 2nd or 3rd occasions) compared to a non-chitosan treatment. A control with no chitosan and no ethylene was included. Data provided by ASSAM- Centro operativo di agrometereologia, Agenzia Servizi al Settore Agroalimentare delle Marche, wether station of Montegiorgio).

The field of the experiment had the following crops before the experiment: durum wheat (harvest year Species yield): 2012 durum wheat 5.4 t ha^{-1}; 2013 barley 5.8 t ha^{-1}; 2014 rapeseed for seed production 1.0 t ha^{-1}; 2015 durum wheat 5.5 t ha^{-1}; 2016 coriander for seed production 1.3 t ha^{-1}; 2017 onion for seed production 1.5 t ha^{-1}; 2018 durum wheat 4.9 t ha^{-1}.

After the wheat harvest in June 2018, the wheat straw was removed, and the soil was harrowed at 40 cm depth on 4 August 2018. On 4 October 2018 and 10 October 2018, the soil was harrowed at 20 cm depth and milled at 10 cm, respectively.

The transplant of the bulbs was carried out on 13 October 2018 with bulbs of two different lines, male line, used as a pollinator, and the female line, male-sterile, from which the seed will then be collected. The arrangement was to alternate 8 female lines and 4 male lines. The bulbs had an average weight of 32 g and were laid in the soil with a special transplanting machine, at a density of 27.4 bulbs m^{-2}, with rows 70 cm apart.

Sprouting occurred about 20 days after transplantation (DAT), with homogeneous uniformity for both the male and female lines.

The list of fertilizer and active ingredients applied and relative quantities of the product are reported in Table 1.

Table 1. List of the products (fertilizes and active ingredients) applied in the experimental onion field, along with date of application, number of products and, for the nutrients, amount of nutrients per unit area. The author does not endorse or sponsor any commercial product, service, or activity.

Date (DD/MM/YYYY)	Management Technique	Active Ingredient/Formulation	Product [kg or L ha^{-1}]	N [kg ha^{-1}]	P_2O_5 [kg ha^{-1}]	SO_3 [kg ha^{-1}]
26 September 2018	Herbicide	Glyphosate	2.0 L	-	-	-
14 October 2018	Herbicide	Pendimetalin	1.5 L	-	-	-
17 October 2018	P fertilization	Single superphosphate	500 kg	-	230	
7 November 2018	Localised starter fertilization	Gran verde top start 8 N-35 P (including 5% SO_3 and 0.8% Zn)	18.8 kg	1.5	6.6	-
4 December 2018	Fungicide	Copper oxychloride	3.10 kg	-	-	-
7 February 2019	N fertilization	N [35 N-23 SO_3]	437.5 kg	153.1	-	100.6
26 February 2019	Fungicide	Copper oxychloride	1.5 kg	-	-	-
9 March 2019	Fungicide	Iprovalicarb + Copper oxychloride	1.3 kg	-	-	-
19 March 2019	Fungicide	Mancozeb + Zoxamide	1.25 kg + 0.63 L	-	-	-
1 April 2019	N fertilization	N [35 N-23 SO_3]	187.5	65.6	-	43.1
19 April 2019	Fungicide	Metalaxil + Dimetomorf + Pyraclostrobin	2.5 kg + 2.5 L	-	-	-
3 May 2019	Fungicide	Chlortalonil + Metalaxyl	1.88 L	-	-	-
6 May 2019	N fertilization	Ammonium nitrate 26 N	187.5 kg	48.75	-	-
23 May 2019	Fungicide	Zoxamide + Cimoxanil	0.63 L + 0.38 kg	-	-	-
8 June 2019	Fungicide	Dimetomorf + Pyraclostrobin	2.5 L	-	-	-
26 June 2019	Fungicide	Thiophanate-methyl	1.25 L	-	-	-

2.2. Experimental Device in the Field

The trial was an unbalanced randomized block design with 4 replicates. Each plot had an area of 2.25 m^2: 1.5 m wide, comprising two onion rows of 2 m length each. In total,

32 plots were established. All treatments and measurements were performed exclusively on the female (i.e., the male-sterile) line.

The ethylene treatments (dose and timing) applied included:

- Recommended rate = 100% applied at early application (21 March 2019), hereafter referred as 1st Et100 2nd Et0 3rd Et0. At the time of the application, the crop was at the phenology stage 306 of the BBCH scale [36];
- Rate = 150% compared to the recommended, applied as 100% at early application, as above + additional 50% at mid- or late applications, carried out on 2 April 2019 and 28 April 2019, respectively. In these dates, the phenology stages 402 and 501 of the BBCH scale [36], respectively. These treatments were referred as 1st Et100 2nd Et50 3rd Et0 and 1st Et100 2nd Et0 3rd Et50, respectively;
- Recommended rate = 100% applied at late application (28 April 2019), hereafter referred as 1st Et0 2nd Et0 3rd Et100;
- Control (ethylene never applied), referred as 1st Et0 2nd Et0 3rd Et0.

Treatments receiving the 100% rate in the early application occasion and 150% rate were also treated or not with chitosan (referred as with and without chitosan, respectively).

Ethylene was applied as ethephon with sprayers. During the application, the control plots were covered with special plastic material to avoid contamination.

Rates of the 100% recommended rate applied in the 1st application included the application of 1 L ha^{-1} of a commercial product (Ethrel®, Bayer, containing Etefon at a rate of 39.6%. The author does not endorse or sponsor any commercial product, service, or activity). An amount of Etefon of 39.6 g (density = 480 g/L) was dissolved in 300 lt of water and distributed with sprayer bar; the dose of etephon used is 46.125 g/ha (100% rate).

In the second and third applications, the distribution was carried out manually with a hand sprayer: 15 mL of Ethrel® (Bayer. The author does not endorse or sponsor any commercial product, service, or activity) was dissolved in 11 L of tap water, then 200 mL of solution was distributed for each plot in the 50% rate. In the 100% rate at 3rd application, 30 mL of commercial product were used. In all conditions, control plots were treated with identical amount of water without ethylene.

Application of chitosan in the 'with chitosan' plots was carried out weekly between 8 May 2019 and 3 June 2019 (phenology stages 505 to 509 of the BBCH scale [36]). To do so, 150 g of chitosan (Chitosano, Agrilaete, Italy, 100% a.i.) were dissolved in 15 L of tap water and sprayed manually through a hand pump. The chitosan suspension was prepared 12 h before application. Plots 'without chitosan' received an identical amount of tap water only.

2.3. Measurements before and after the Application of the Ethylene and Chitosan

During the crop growth, the following data were collected on each plot:

- Number of bulbs and culms per row at the stage of 3 leaf stage (phenology stage 103 of the BBCH scale [36]);
- Number of inflorescences per row at full flowering phase (phenology stage 605 of the BBCH scale [36]);

At the phenology stage 905 of the BBCH scale [36], the infructescence scapes were manually collected by cutting 7–10 cm below the fruits, and they were laid in a greenhouse to dry for 20 days and then manually threshed in September 2019. Seed yield per plot and 1000 seed weight (on 2 subsamples of 400 seeds, each) were recorded. Number of seeds per inflorescence was computed.

2.4. Germination Test

Total germination was measured on a fresh subsample of seed, and germination trend of aged seed was carried out in January 2021 on seed stored at 15 °C from harvest to test [37].

Seeds were washed in distilled water and soaked in 4.5% sodium hypochlorite in distilled water for 5 s. After soaking, seeds were rinsed with distilled water 3 times. Sixty

seeds were laid out in each petri dish containing a filter paper. Petri dishes and filter paper were previously sterilized under UV rays for 45 min.

Each plate was kept moist with distilled water by adding an amount of water needed to completely soak the filter paper. The plates were kept under natural light at 22 °C of constant temperature. At regular intervals of time, the germinated seeds were removed, and the filter paper was refilled with distilled water when needed. The test was ended when no germination occurred for 2 consecutive weeks.

2.5. Computations and Statistical Analyses

The experimental design was unbalanced and consisted of two factors: application of Ethylene in various phenological phases (i.e., timing) and amounts (referred as "Treat") and application of chitosan nested into the Ethylene application treatment (referred as "Chit (Treat)").

Data on grain yield, yield components and final germination were analyzed with a general linear mixed model (Glimmix procedure in SAS/STAT 9.2 statistical package; SAS Institute Inc., Cary, NC, USA). The model used was specifically built for unbalanced designs. See Refs. [38–40] for details on the procedure and the SAS procedure applied.

In particular, the chitosan application was nested into ethylene application. Block was added as a random factor.

The analyses were performed included by including unbiased estimates of variance and covariance parameters assessed by restricted maximum likelihood (REML). Denominator degrees of freedom of each error were estimated by Kenward–Roger approximation (according to which null covariance parameters do not contribute to degrees of freedom of the model) and interaction-specific error terms. Least square means (LSmeans) of the treatment distributions were computed. Differences among LSmeans were compared by applying Tukey–kramer grouping at the 5% probability level to the LSMEANS p-differences. When denominator degrees of freedom were not constant and in the presence of heteroscedasticity, "ADJDFE = ROW" statement was used to adjust for multiple comparisons. LS-Means and their standard error estimates per treatment are provided in Supplementary Materials Table S1.

Data on the pattern of germination were analyzed with 3 different strategies. Data on cumulative germination at increasing the time from sowing in plate were treated with a GLIMMIX procedure as above. In the Glimmix procedures, Strategy 1 included the inclusion of the effect of time as a class variable with no parameter estimation after application of a heterogeneous autoregressive covariance structure to the time of sampling to take into account the repeated measurements as applied in [41]. Strategy 2 included the inclusion of time as a continuous variable with all treatments (either continuous or class) parameter estimation through the application of the 'solution' option in the model statement. Strategy 2 also included a Cumulative Logit link function and a Diagonal Variance Matrix. Additional strategies, including Laplace pseudo-likelihood approximation, were tested and discarded after checking for the model fitting options.

Strategy 1 was thus used to achieve LSmeans estimate of the time effect and check for shrinkage compared to the arithmetic means. Strategy 2 was used to achieve the parameter estimates. Strategy 1 achieved better model fitting parameters compared to Strategy 2. In both strategies, only "time" showed a $p < 0.05$, and the other factors applied showed $p > 0.1$. Thus, the results of Strategy 2 were provided in Supplementary Table S2, and Strategy 1 was retained.

Nonetheless, the variation by time was modeled by the slidewrite program applying a generalized logistic regression (i.e., a sigmoid function) with four parameters (Strategy 3) after imposing the origin [0;0] as an intercept (i.e., 0% germination at the time 0). See Refs. [42,43] for details. Confidence intervals at 95% of the parameters and confidence and prediction intervals of the sigmoid model were computed, along with its R^2 and fit statistics to the observed data.

3. Results

3.1. Stand Traits and Environmental Conditions during the Growing Season

The growing season was slightly warmer in the late winter and colder in the early spring compared to the long-term average (Figure 1). The growing season was characterized by low rainfall between the end of winter and the beginning of spring, especially in February and March, where rainfall was 32.8 mm and 57.9 mm, respectively. The month of May, during which the plant started flowering, was particularly rainy and cool compared to the 2000–2019 average.

3.2. Grain Yield, Yield Components, and Germination

The influence of the ethylene and chitosan treatments was evident on the seed yield and the flower fertility (i.e., seeds per inflorescence at seed maturity) (Table 2), whereas the ethylene application, but not chitosan, affected the thousand seed weight. No effects of the treatments were found on the number of inflorescences at full blooming and the final germination of the aged seed.

Table 2. F and *p*-values of the general linear mixed model applied to the onion grain yield, yield components and final germination. Factors were the moment and amount of ethylene applied [Treat] and the Chitosan nested in the ethylene application [Chit(Treat)]. Moment of ethylene application included an early, mid, and late applications indicated as first, second and third (1st, 2nd, and 3rd, respectively) occasions in the figures. Amount of ethylene application included 100% recommended rate (in the 1st or 3rd occasions) or 100% in the 1st + 50% in either the 2nd or 3rd occasions. Chitosan application was performed in "Treat" including a 100% application in the 1st occasion (irrespective of the further 50% application in the 2nd or 3rd occasions) compared to a non-chitosan treatment. A control with no chitosan and no ethylene was included. Values at $p < 0.05$ were indicated in bold. Numerator degrees of freedom are indicated once and apply to all variables, denominator degrees of freedom were estimated by the Kenward Roger approximation. LS means and their standard error estimation are provided in Supplementary Material Table S1.

	Effect	**Treat**	**Chit (Treat)**
	Num DF	**4**	**3**
Seed Yield [g m^{-2}]	**Den DF**	**24**	**24**
	F	**5.59**	**7.76**
	p	**0.0025**	**0.0009**
Inflorescence at full blooming [n m^{-2}]	**Den DF**	20.12	20.17
	F	0.57	0.19
	p	0.6873	0.9012
Seeds per Inflorescence at seed maturity	**Den DF**	**20.15**	**20.24**
	F	**6.47**	**9.27**
	p	**0.0016**	**0.0005**
Thousand seed weight [g]	**Den DF**	**20.39**	20.66
	F	**5.69**	1.04
	p	**0.0031**	0.3938
Final germination [%]	**Den DF**	21.75	22.03
	F	1.2	0.54
	p	0.3406	0.6578

Differences among treatments for the seed yield (Figure 2) strongly depended on both the amount and time of ethylene application: in particular, no differences occurred between the distribution of ethylene at full dose (100%) in the third application, without application of chitosan, compared to the control (no ethylene application and no chitosan). Furthermore, differences between these latter two treatments and the ethylene applied at the first application (either with or without chitosan) were unclear and detected by the conservative grouping applied, despite the first application appearing to slightly, but inconsistently, reduce the seed yield (−8.6% compared to the control). In contrast, applying

a 150% dose of ethylene without chitosan strongly reduced the seed yield irrespective of the time of the additional 50% application (either the second or third application, by 34.1% and 40.7% compared to the control). In these two latter treatments, the chitosan application stimulated yields irrespective of the time of the additional 50% ethylene application.

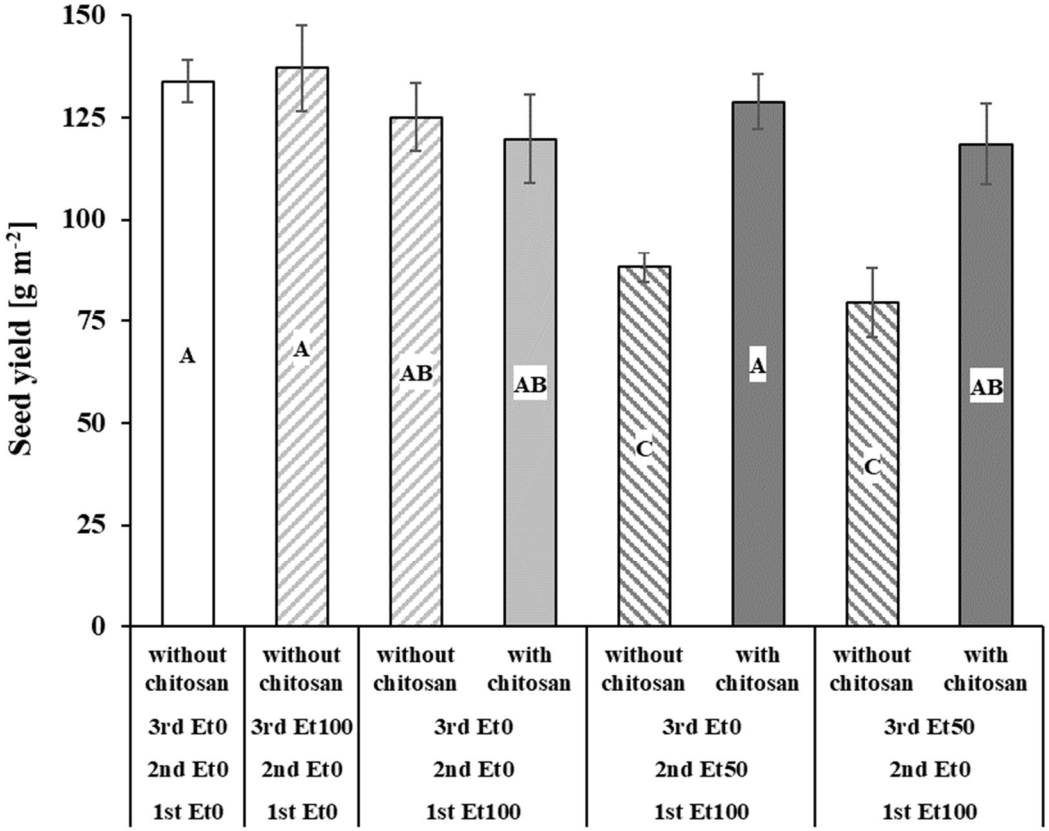

Figure 2. Role of the Ethylene [Et] application at full dose [100%, indicated as Et100] or half dose [50%], indicated as Et50 or no application [0%, indicated as Et0] during the first, second and third (1st, 2nd, and 3rd, respectively) occasion as shown in Figure 1) and chitosan application on the Seed yield of onion growing under rainfed conditions. Moment of ethylene application included an early, mid, and late applications indicated as first, second and third (1st, 2nd, and 3rd, respectively) occasions in the figures. Amount of ethylene application included 100% recommended rate (in the 1st or 3rd occasions) or 100% in the 1st + 50% in either the 2nd or 3rd occasions. Chitosan application was performed in "Treat" including a 100% application in the 1st occasion (irrespective of the further 50% application in the 2nd or 3rd occasions) compared to a non-chitosan treatment. A control with no chitosan and no ethylene was included. When Chit(Treat) showed a $p < 0.05$, p-differences of the LSmeans were computed and separated by a conservative Tukey − Kramer grouping at the 5%. Bars with a letter in common should not be considered different according to Tukey − Kramer. Data are arithmetic means and standard errors ($n = 4$).

The reduction of the seed yield in the 150% ethylene application treatments matched with those in the fertility of the inflorescence (measured as the number of seeds per inflorescence, Figure 3). Indeed, no differences in the distribution of ethylene at full dose were carried out either in the first or third application, with or without chitosan, compared to the control. Similarly to the seed yield, applying 150% ethylene reduced the number of seeds per inflorescence by 30.2% and 36.7% when the additional 50% dose was applied in the second or third application, respectively.

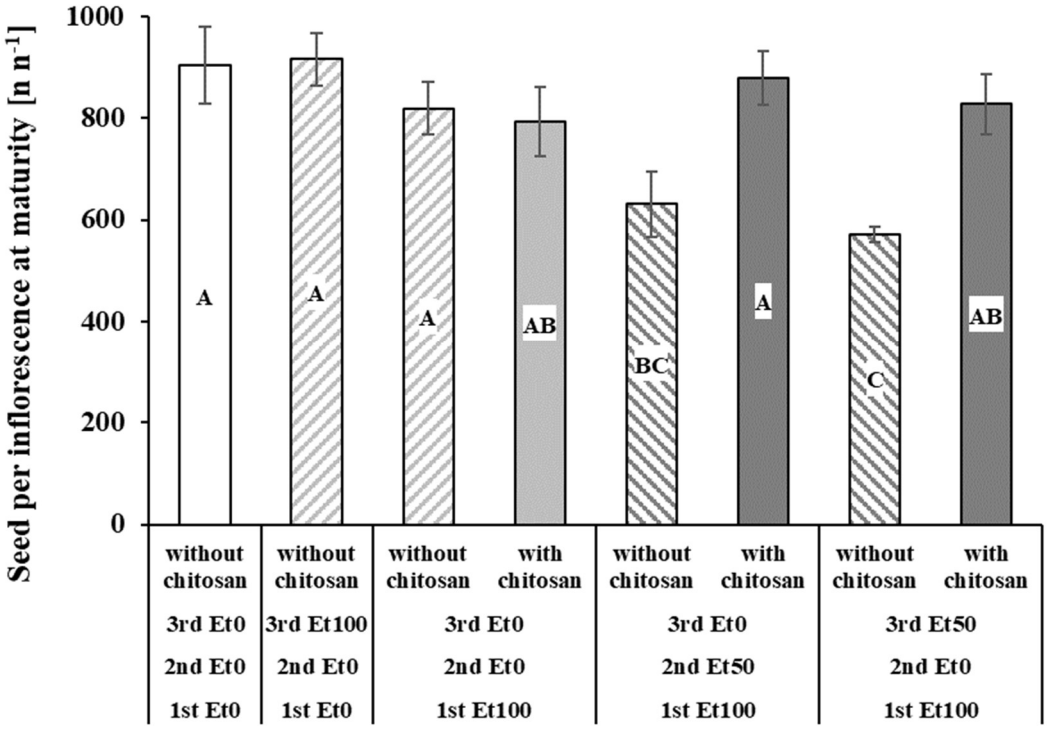

Figure 3. Role of the Ethylene [Et] application at full dose [100%, indicated as Et100] or half dose [50%], indicated as Et50 or no application [0%, indicated as Et0] during the first, second and third (1st, 2nd, and 3rd, respectively) occasion as shown in Figure 1) and chitosan application on the number of seeds per inflorescence of onion growing under rainfed conditions. Moment of ethylene application included an early, mid, and late applications indicated as first, second and third (1st, 2nd, and 3rd, respectively) occasions in the figures. Amount of ethylene application included 100% recommended rate (in the 1st or 3rd occasions) or 100% in the 1st + 50% in either the 2nd or 3rd occasions. Chitosan application was performed in "Treat" including a 100% application in the 1st occasion (irrespective of the further 50% application in the 2nd or 3rd occasions) compared to a non-chitosan treatment. A control with no chitosan and no ethylene was included. When Chit(Treat) showed a $p < 0.05$, p-differences of the LSmeans were computed and separated by a conservative Tukey − Kramer grouping at the 5%. Bars with a letter in common should not be considered different, according to Tukey − Kramer. Data are arithmetic means and standard errors ($n = 4$).

A dose of 100% ethylene, distributed in the first application (when the crop was at the early stem elongation stage), allowed to have a strong increase in the weight of a thousand seeds (+3.2% on average compared to the control, Figure 4). In contrast, inconsistent differences in the thousand seed weight were found between the control, from one side, and the treatments, including the 150% ethylene dose.

The number of bulbs at full emergence and the number of culms at full emergence were analyzed to check for differences in the plots within treatments since the treatments were established after the bulb planting and culms differentiation. These variables did not change according to the treatments (Supplementary Material Table S1); thus, culms per bulb at full emergence did not occur, and in the inflorescence per Bulb at full blooming, and seed yield per bulb depended on the number of inflorescence per unit area and seed yield, respectively.

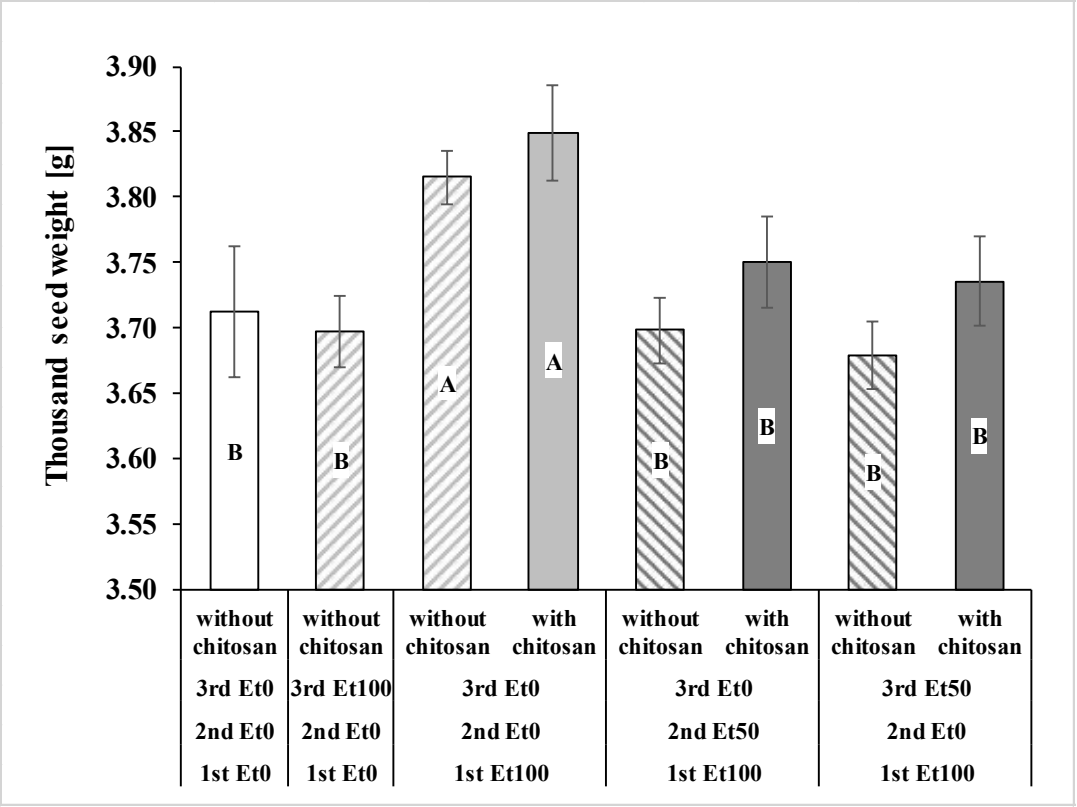

Figure 4. Role of the Ethylene [Et] application at full dose [100%, indicated as Et100] or half dose [50%], indicated as Et50 or no application [0%, indicated as Et0] during the first, second and third (1st, 2nd, and 3rd, respectively) occasion as shown in Figure 1) and chitosan application on the thousand seed weight of onion growing under rainfed conditions. Moment of ethylene application included an early, mid, and late applications indicated as first, second and third (1st, 2nd, and 3rd, respectively) occasions in the figures. Amount of ethylene application included 100% recommended rate (in the 1st or 3rd occasions) or 100% in the 1st + 50% in either the 2nd or 3rd occasions. Chitosan application was performed in "Treat" including a 100% application in the 1st occasion (irrespective of the further 50% application in the 2nd or 3rd occasions) compared to a non-chitosan treatment. A control with no chitosan and no ethylene was included. Since Treat, but not Chit(Treat), showed a $p < 0.05$, p-differences of the LSmeans were computed and separated by a conservative Tukey–Kramer grouping at 5%. Letters displayed were thus rebuilt from the "Treat" LSmeans as shown in Supplementary Material Table S1. Bars with a letter in common should not be considered different, according to Tukey–Kramer. Data are arithmetic means and standard errors ($n = 4$).

3.3. Cumulative Germination

The average final germination of the aged seed was 78%, with no differences among the treatments applied (Table 2, see Supplementary Material Table S1 for the final germination data and Supplementary Material Table S2 for the parameter estimation).

Among the fixed factors applied, the time, but not the treatments, showed an effect on the temporal variation of the germination (Table 3). In particular, the intercept was negative (indicated by the Beta parameter from the sigmoidal function fitted in Figure 5 and Table 4 and by the intercrop estimate in the general linear mixed model provided in the Supplementary Material Table S2).

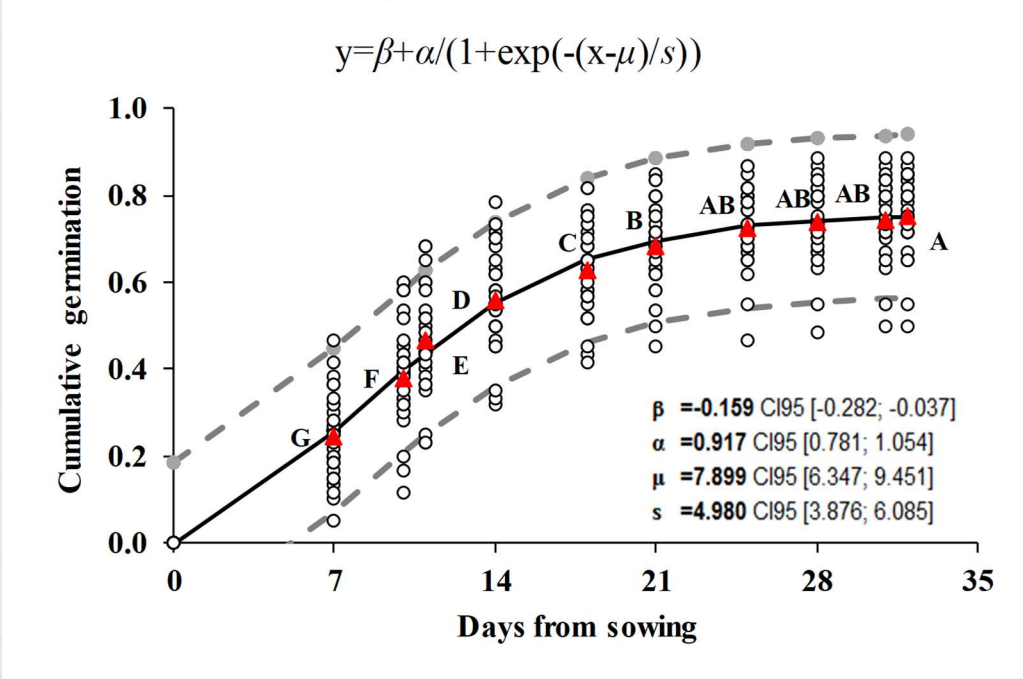

Figure 5. Mean variation by time of the cumulative germination of the seed of onion. Nor the Ethylene application or chitosan showed to affect the regression, thus, raw data (white circles) per sampling occasion were used. Red triangle indicate the LSmeans p-difference separation of the Time effect in Table 3 computed and separated by a conservative Tukey − Kramer grouping at the 5%. LSMeans with a letter in common should not be considered different according to Tukey − Kramer. Data are arithmetic means and standard errors (n = 4). The model fitted is indicated with a solid black line and prediction intervals at the 95% were shown (gray dashed lines). The sigmoid function fitted to the time variation is shown as a pic title and its coefficient embedded. Analysis of the sigmoidal function fitted is reported in Table 4.

Table 3. F and p-values of the general linear mixed model applied to the temporal trend of the cumulative germination of onion seed. Factors were the time (referred as "Time" and included as a class variable), the moment and amount of ethylene applied [Treat] and the Chitosan nested in the ethylene application [Chit(Treat)] and interaction terms [Treat × Time, and Chit × Time (Treat)]. Values at $p < 0.05$ were indicated in bold. Numerator degrees of freedom are indicated, denominator degrees of freedom were estimated by the Kenward Roger approximation.

Effect	Num DF	Den DF	F	p
Time	**9**	**60.35**	**243.52**	**<0.0001**
Treat	4	20.31	0.63	0.6449
Chit(Treat)	3	20.31	0.6	0.6225
Treat × Time	36	99.8	0.55	0.9784
Chit × Time (Treat)	27	93.43	0.56	0.9556

Table 4. Function fitted, determination coefficient (either raw or adjusted) and estimation of the coefficients of the sigmoid shown in Figure 4 and *p*-values of the general linear mixed model applied to the temporal trend of the cumulative germination of onion seed. Factors were the time (referred as "Time" and included as a class variable), the moment and amount of ethylene applied [Treat] and the Chitosan nested in the ethylene application [Chit(Treat)]. Values at $p < 0.05$ were indicated in bold. Numerator degrees of freedom are indicated, denominator degrees of freedom were estimated by the Kenward Roger approximation.

Function Fitted *		**r2 Coef Det**	**DF Adj r2**	**Fit Std Err**	**F-Statistic**
$y = \beta + \alpha/(1 + \exp(-(x - \mu)/s))$		0.86	0.86	0.09	727.49
Coefficient	**Value**	**Standard error**	**t-Value**	**lower 95% Confidence Limits**	**upper 95% Confidence Limits**
b	−0.16	0.06	−2.59	−0.28	−0.04
α	0.92	0.07	13.29	0.78	1.05
μ	7.90	0.78	10.08	6.35	9.45
s	4.98	0.56	8.93	3.88	6.08
Source	**Sum of Squares**	**df**	**Mean Square**	**F-Statistic**	
Regression	19.46	3	6.49	727.49	
Error	3.10	348	0.01		
Total	22.57	351			

* please note that in contrast to the analysis reported in Table 3, the 0,0 intercept was imposed in the present analysis.

The final germination occurred 25 days after the beginning of the germination test, with no further detectable increase in the germination until the end of the test. The variation of the germination did not occur homogeneously with time. Half of the seeds germinated before 7.9 days from sowing, despite the germination occurring relatively homogenously, as indicated by the average s-parameter in Table 4.

4. Discussion

The seed yield of hybrid onion can vary according to the male-fertile to male-sterile (F:S) plant ratio, in addition to a wealth of other agronomic and environmental conditions (e.g., nutrients, temperatures, water availability, etc.). In the present work, we used an F:S = 4:8, which was found to not limit the pollination activity and the seed set [44,45]. In addition, the strong diversification around the field and the presence of nearby bee hives [46,47] likely allowed for the presence of pollinators. We thus assume that no pollination limitation may have occurred in the field.

In the present work, the average yield across treatments was 1164 ± 249 kg seed ha^{-1} (mean ± standard deviation), which was higher than the maximum yield with optimal conditions in other works [17,48]. Nonetheless, we cannot exclude that the drought in the critical phases of the crop development, and in particular during the seed bolting, may have reduced the yield potential, as also shown by El Balla et al. [49].

Ethylene is involved in the senescence of most flowers; it can anticipate the flower emission and affect the temporal extent of the flower's ability to be pollinated and efficiently making the fruit setting, and, in addition, it can stimulate the plant production under suboptimal conditions, including nutrient shortage or other abiotic stresses, while supporting the plant K starvation occurs and allowing for a longer opening of the stomata under water stress [27,50–52], which also makes it an efficient tool in the crop management. Thus far, the crop response to the ethylene application in terms of yield or physiological traits strongly depends on the time and amount applied and is affected by the genotype × environment interaction.

In the present study, we found that application in the field at the recommended dose (100%) did not affect the seed yield of onion, and this occurred with both the early (first) and late (third) occasion of application. In contrast, when an additional 50% of ethylene was applied on the second or third occasion (for a total of 150% compared to the recommended dose), a strong decline in the seed yield was found.

The role of ethephon and ethylene-based commercial products were studied in many species, including onion and other Alliaceae. Usually, ethylene inhibits the growth of young leaves, regulating both cell expansion and cell growth [53]. Ethylene was found to have a depression activity on various botanical fractions and physiological parameters in onion, including a strong reduction in total biomass, the scape, and the chlorophyll concentration regardless of its endogenous or exogenous origin [54–57], while at the one hand increasing the concentration of various metabolite [55], which were found to be directly related to various stresses in the crops [22,23,38,58], and the transpiration rate including under drought stress conditions [56]. The reaction of monocots in terms of seed yield to biomass reduction can strongly vary. In wheat, seed yield increases while reducing plant height and straw biomass, and this occurs thanks to an improved transfer of photosynthates from the shoots to the flowers [59]. Thus far, this occurs given that biomass reduction (modulated in wheat by the Rht genes) does not depress the net photosynthesis. A similar genetic control exists in onions [54]. In our experiment, the 150% ethylene application may have contributed to reducing the mass of vegetation or the photosynthetic activity in the active growth phase near or during the fruit set phase. At the same time, the multiple application of ethylene in both the 150% recommended doses may have reduced the total seed yield, as found by Thomas and Rankin [60] when both are applied as a low dose in multiple applications or a high dose in either one or multiple applications in two onion genotype: a hybrid, such as in the present study, and an open-pollinated genotype. Furthermore, we cannot exclude that the genotype used in the present study may not be able to efficiently regulate the photosynthesis under high ethylene application, as also shown in tobacco and Arabidopsis [61].

In contrast, the application of lower ethylene concentration compared to the present study stimulated the seed yield [32]. The latter authors (Singh et al. [32]) also found that ethylene stimulated yield through an increase in the number of inflorescence per unit area. In our study, we showed that the yield depression under high doses of ethylene application mostly occurred through a depression of the flower fertility (measured as the number of seeds per inflorescence), so that the later application of ethylene (i.e., the additional 50% doses) may have strongly affected the process of the seed fertilization, as showed in Arabidopsis by Völz et al. [62]. On the one hand, the positive effects of ethylene on female flower production were mostly seen in dicots [63], whereas many species were shown to block or delay the flowering or shorten the flower life after high exposure to ethylene [27]. Similarly, we showed that ethylene stimulated the seed size (measured as a thousand seed weight), which is a consequence of a reduced grain number, as shown in Huges et al. [64].

In our experiment, the increase in yield in high-ethylene (150%) plots treated with chitosan compared to the high-ethylene (150%) plots without chitosan may thus be a consequence of the chitosan effects on the reduction of the transpiration. In particular, chitosan was shown, in addition to the biotic stress control effects, to stimulate the closure of the stomata and thus limit the loss of water by transpiration of plants through the formation of a coating on the leaves [65–67] and other resistance mechanisms to stresses [68], which were likely important in our experiment given the high evapotranspiration demand during fruit set. Indeed, we cannot exclude that chitosan may have, on the one hand, stimulated the abscisic acid, whose activity can compete with ethylene, since such stimulation was hypothesized [69]. Furthermore, chitosan may have provided an improvement in the onion's resistance to fungal pathogens [70]. Nonetheless, the crop was treated with fungicides and did not show either any visible sign of pathogenic or insect attack.

Given the higher seed size in the ethylene-treated plots, especially in the 100% rate (either as early or late application), we expected to find an increased germinability of the seed. Nonetheless, no differences among treatments were found in both the final germination and the germination rate. Orsini et al. [57], in an experiment from the same environment and conditions, found augmented germination of the freshly collected seed after the ethylene application to the plant [57]. Nonetheless, Orsini et al. [57] also observed a higher seed yield and lower seed size than the present study, so the stimulation of the

seed germination ability after the application of the ethylene to the plant may depend on the seed size. Similarly, El Balla et al. [49] found that smaller onion seeds than the present study may experience a reduction of germination when experiencing drought stresses, also highlighting that such reduction may strongly depend on the environmental conditions. In addition, we found that our mean germination rate was similar to the ethylene-treated plants in Orsini et al. [57], thus suggesting that further increases in seed germination rate may be hard to achieve. In this work, the germination of the freshly collected seed does suggest that the collection period allowed to have mature seeds with mature embryos, as seen in Spurr et al. [71]. Furthermore, the present ethylene application may have been too low to produce and affect the germination [72]

Aging the seed at 15 °C in the dark for 17 months after harvest reduced the final germination by around 14%. Such a loss of germination highlights the ability of the seed lot from our experiment to maintain over time a good ability to germinate. Thirusendura Selvi and Saraswathy [4] showed that the storage conditions used in the present study (natural drying and storage at 15 °C at low relative humidity) are only partially necessary to preserve the vigor and viability of the seed. Indeed, we found that, on average, 50% of germination occurred before 8 days (μ in the sigmoid model function of germination built), with almost no delay time at the beginning of the germination (i.e., a low μ and a high s parameter in the sigmoid describing the germination pattern, but see the Supplementary Material Table S3 to easily surf among on these parameters). These patterns have been found in untreated and fresh (i.e., unaged) orthodox seeds [42,43], and similar germination and early growth patterns were found in onions subjected to mechanical impedance [73] and optimal temperature and water availability [12,74,75], the latter of which found, in the field, similar germination time compared to our experiment in the plate. The present data agree with other experiments, including naturally or artificially aged seeds [76,77].

5. Conclusions

In conclusion, high doses of ethylene from the present study strongly depressed the yield, and this was likely due to a stimulus of the transpiration under drought, whereas the recommended dose did not increase the yield while increasing the seed size. Such increase was, however, unrelated to the germination ability of the seed, which maintained a relatively high and fast germination rate even under natural aging. The application of the chitosan exerted a biostimulant effect and increased the yield under the low yield potential.

The present results are important to define both the amount and timing of ethylene application to improve the seed production of the onion.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8090781/s1, Table S1: results of the statistical analysis, LSmeans, their standard error estimates and Tukey–Kramer grouping of the variables measured and computed; Table S2: statistical model of the germination built and parameters estimates by the GLMM; Table S3: table for building explanatory sigmoid functions.

Author Contributions: Conceptualization, S.S. and G.R.; methodology, D.V. and S.S.; software, S.S.; validation, S.S. and M.M.; formal analysis, S.S.; investigation, D.V., F.G.S.A. and S.S.; resources, D.V., G.R., M.M. and S.S.; data curation, S.S.; writing—original draft preparation, D.V., F.G.S.A. and S.S.; writing—review and editing, G.R., M.M. and S.S.; visualization, F.G.S.A. and S.S.; supervision, S.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are grateful to ASSAM- Centro Operativo di Agrometereologia, Agenzia Servizi al Settore Agroalimentare delle Marche for providing the meteorological data.

Conflicts of Interest: The authors declare no conflict of interest. The author does not endorse or sponsor any commercial product, service, or activity.

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