Improving seed germination of the eggplant rootstock *Solanum torvum* by testing multiple factors using an orthogonal array design

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ABSTRACT

Solanum torvum is a highly vigorous relative of eggplant that is resistant to a number of harmful soil-borne diseases and is compatible for grafting with eggplant. Being a potential rootstock, this plant frequently presents poor and erratic germination, which makes its practical use difficult. We used an L8 (2^7) orthogonal array design to evaluate the primary effects of seven factors (soaking of seeds, scarification with sodium hypochlorite (NaClO), application of gibberellic acid (GA_3), use of potassium mitrate (KNO_3) as a moistening agent, cold stratification, application of a heat shock, and light irradiation during germination) at two levels (L0 and L1) using four germination parameters (early and final germination, germination rate and vigour index) in fresh *S. torvum* seeds. *Solanum torvum* seeds had a strong dormancy with no germination in the untreated seeds and high early and final germination (approximately 100%) in certain treatments. An evaluation of the main effects revealed highly positive effects on germination from seed soaking, and the use of GA_3, KNO_3, and light irradiation, whereas NaClO scarification had a negative effect. The application of cold stratification and heat shock treatments also had a positive effect on seed germination but to a lesser extent than the other treatments. An improved proposed protocol that consisted of subjecting seeds to soaking, the application of GA_3 and KNO_3, cold stratification, heat shock, and light irradiation was validated and demonstrated to be highly effective, with seed germination success greater than 60% being observed at 3 d and final germination reaching a plateau at 6 d. A second validation experiment using a commercial growing substrate also showed a high emergence (approximately 50%) at 7 d and a final germination of approximately 80% was recorded with application of the improved protocol. The seed germination protocol that we have developed will facilitate the use of *S. torvum* as a rootstock for eggplant and its use in breeding programmes. Our results also reveal that orthogonal array designs are a powerful tool for establishing improved protocols for seed germination.

Keywords: Solanum torvum, seed, germination, dormancy, orthogonal arrays, main effects

1. Introduction

Solanum torvum Sw., commonly known as turkey berry, devil's fig or pea eggplant, is a wild bush of neotropical origin that belongs to the "spiny *Solanum*" (subgenus *Leptostemonum*) group (Levin et al., 2006). This species has become naturalised and is sometimes invasive in tropical areas of Africa, Asia, and Australia; also, this species is occasionally cultivated, primarily in Southeast Asia and Africa, for its edible fruits (Gousset et al., 2005; Nyadanu and Lowor, 2015). *Solanum torvum* is of great interest as a rootstock for eggplant (*S. melongena* L.), as the plant is highly vigorous, fully graft-compatible with eggplant scions (Gisbert et al., 2011b; Moncada et al., 2013), and possesses resistance to a wide range of soil pathogens, such as *Verticillium dahliae*, *Ralstonia solanacearum*, *Fusarium oxysporum*, and root-knot-nematodes (Bletsos et al., 2003; Gousset et al., 2005; Bagnaresi et al., 2013), as well as being tolerant to abiotic stresses (Schwarz et al., 2010). Furthermore, eggplant fruits produced on scions grafted onto *S. torvum* are of good quality (Gisbert et al., 2011b; Moncada et al., 2013; Miceli et al., 2014). Additionally, grafting eggplant on *S. torvum* reduces translocation of the heavy metal cadmium (Cd) from the roots to the aerial part (Arao et al., 2008) and may minimize the negative effects on the fruit quality from Cd soil contamination (Savvas et al., 2010). Because of these desirable traits, *S. torvum* also represents a genetic resource of strong relevance to the introgression breeding of eggplant (Kumchai et al., 2013)

The primary limitation for the practical use of *S. torvum* as a rootstock in the commercial production of grafted eggplant plants, as well as in breeding programmes, is the poor, irregular and erratic germination due to dormancy in seeds (Ginoux and Laterrot, 1991; Miura et al., 1993; Gousset et al., 2005; Hayati et al., 2005). This characteristic has even led to the proposed use of vegetative propagation to overcome the seed germination problem (Miceli et al., 2014). The breaking of dormancy, which is a common phenomenon among wild *Solanum* species (Taab and

Andersson, 2009; Wei et al., 2010; Kandari et al., 2011; Tellier et al., 2011), and the enhancement of germination can be achieved using combinations of many different physical (e.g., seed soaking, manual scarification, cold stratification, heat shocks, light irradiation, and magnetic fields) and/or chemical (e.g., scarification with acidic or basic chemicals, plant growth regulators, and osmotic treatments) treatments (Finch-Savage and Leubner-Metzger, 2006; Bewley et al., 2013; Holubowicz et al., 2014).

Determining the critical combination of factors that permit the enhancement of germination in *S. torvum* seeds is important to develop improved protocols for seed germination in this species for its use as a rootstock and for breeding purposes (Hayati et al., 2005; Gisbert et al., 2011a). The influence of several potentially key factors affecting a variable, in this case seed germination, can be determined by studying one factor at a time (as achieved by Hayati et al., 2005). However, this process greatly reduces efficiency when there is an interdependency of factors or when it is impractical to isolate and test each variable individually. Full factorial designs, which are much more efficient in determining the optimal combination of factors, may require large and costly experiments when many factors are involved (Onyiah, 2008; Rao et al., 2008). An alternative commonly used in industrial applications are orthogonal (Taguchi) arrays (Roy, 2010), which allow the main effects of a large number of factors to be estimated with a limited number of treatments. Although orthogonal arrays have been successfully applied to address the problem of determining adequate combinations of factors in biological and biotechnological processes (Rao et al., 2008; Assemi et al., 2012; Sedghi et al., 2014; Vasilev et al., 2014), their use for establishing seed germination protocols has been highly limited (Wu et al., 2011; Poinapen et al., 2013) and largely overlooked.

In this work, we evaluate the primary effects of seven factors potentially involved in the release from dormancy and enhancement of seed germination in dormant seeds of *S. torvum* using an orthogonal array experimental design. The improved protocol established according to the results was then tested and validated. The results from this work will provide information for improving

101 the seed germination of *S. torvum*. These findings will also contribute to facilitating its use as a
102 rootstock and as a source of variation in breeding programmes. At the same time, this work aims to
103 demonstrate the potential of orthogonal array designs for establishing efficient seed germination
104 protocols.
105
106 **2. Materials and Methods**
107
108 *2.1. Seed materials and germination conditions*
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110 Fresh seeds of *S. torvum* accession No. 55953 (originally purchased from Sunshine Seeds,
111 Ahlen, Germany) were extracted from physiologically ripe fruits of plants cultivated in an open
112 field at Universitat Politècnica de València (Valencia, Spain). Five-month-old seeds of *S.*
113 *melongena* accession No. BBS-188/B (landrace from Ivory Coast), with high germination values
114 (>90%), were also used as a control for the validation of the improved treatment developed for the
115 germination of *S. torvum* seeds.
116 Depending on the experiment, seeds were germinated in Petri dishes (9.0 × 2.5 cm; Phoenix
117 Biomedical, Mississauga, Ontario, Canada) on a layer of 0.5 cm of embedded hydrophilic cotton
118 covered by filter paper or sown at a depth of 7 mm in plastic pots (9 × 9 × 9.5 cm) containing
119 commercial nursery growing substrate (Neuhaus Huminsubstrat N3, Lassmann-Dellmann, Geeste,
120 Germany). Twenty-five evenly distributed seeds were placed in each Petri dish or pot. Seeds were
121 sown in Petri dishes and pots at the beginning of the experiments (day 0) in a climatic chamber with
122 a 14-h light / 10-h dark photoperiod at 25ºC constant temperature. A light irradiance of 600
123 $mmol \cdot m^{-2} \cdot s^{-1}$ was provided by GRO-LUX F36W/GRO (Sylvania, Danvers, MA, USA) fluorescent
124 tubes. The pots were watered regularly to keep the substrate moistened.
125
126 *2.2. Factors evaluated*

Seven factors, soaking, sodium hypochlorite (NaClO), gibberellic acid (GA_3), potassium nitrate (KNO_3), cold, heat, and light, with two possible levels (level 0, L0; level 1, L1) for each factor were evaluated for their effects on the germination of *S. torvum* seeds. The levels for each factor were as follows:

(a) Soaking: L0 = no soaking; L1 = soaking seeds in water for 1 d.

(b) NaClO: L0 = no NaClO scarification; L1 = NaClO scarification by the immersion of seeds for 10 min in a 1.2% NaClO (SPB, Cheste, Spain) solution followed by the rinsing of seeds with water.

(c) GA_3: L0 = no GA_3 application; L1 = soaking seeds in a 500 ppm solution of GA_3 (Duchefa Biochemie, Haarlem, The Netherlands) for 1 d.

(d) KNO_3: L0 = use of water as a moistening agent (when using germination in Petri dishes) or for watering (when using germination in growing substrate); L1 = use of a 1,000 ppm KNO_3 (Panreac, Montcada i Reixac, Spain) solution as a moistening agent or as a watering solution.

(e) Cold; L0 = no cold stratification; L1 = seed stratification by placing moist seeds already deposited on Petri dishes with a moistening agent or sown in seedling trays within a wet nursery growing substrate at 4ºC for 7 d.

(f) Heat; L0 = no heat shock; L1 = placing moist seeds already deposited on Petri dishes with a moistening agent or sown in seedling trays within a wet nursery growing substrate at 37ºC for 1 d.

(g) Light: L0 = seeds placed in darkness (Petri dishes covered with aluminium foil); L1 = seeds subjected to light irradiation (16 h of light at an intensity of 600 $mmol \cdot m^{-2} \cdot s^{-1}$ / 8 h dark).

The light factor was considered only for experiments involving the evaluation of germination in Petri dishes. For the experiment involving sowing seeds in a commercial substrate, all seeds were covered with a 7-mm layer of substrate.

The factors soaking, NaClO and GA3 were applied before sowing seeds on Petri dishes or in the nursery growing substrate. The factors KNO_3, cold and heat were applied after sowing seeds, but before initiation of the evaluation of germination or emergence (day 0). The light factor was applied at the initiation of the experiment (day 0). Factors were applied one after the other according to the following order: (1) soaking, (2) NaClO, (3) GA_3, (4) KNO_3, (5) cold, (6) heat, and (7) light. As L1 levels for some of the factors involve pre-germination procedures that may last up to 7 days, their application was programmed so that the initiation of the evaluation of germination or emergence (day 0) was synchronized for all treatments of a given experiment (Table 1).

2.3. Traits evaluated

Seed germination was evaluated at 0, 4, 6, 8, 11, 13 and 15 d after the seeds were placed in the germination cabinet (day 0) for the first experiment (Petri dishes germination), which was aimed at determining the levels of different factors for improving the germination of *S. torvum*. For the experiments aimed at validating the improved treatment, the seed germination (Petri dishes) or emergence (growing substrate) was evaluated at 0, 2, 3, 5, 6, 7, 8, 9, 10, 12, 13 and 14 d after the seeds were placed in the germination cabinet (day 0). Seeds were considered germinated when the radicle was 1 mm or greater. Emergence was evaluated by counting germinating seedlings.

The following four parameters were considered for an analysis of variance (ANOVA) statistical evaluation (Ranal and Garcia de Santana, 2006): (a) early germination/emergence (measured at 4 d or 5 d, depending on the germination experiment, and at 7 d for emergence; %); (b) final germination/emergence (measured at 14 d or 15 d, depending on the experiment; %); (c) germination/emergence rate, which determines the potential for a high final germination combined with a rapid germination/emergence, calculated as $(S_1 \times t_1 + S_2 \times t_2 + \ldots + S_n \times t_n) / (t_1 + t_2 + \ldots + t_n)$, where S_n is the cumulative percentage of germinated seeds at germination test n and t_n is the number of days at which test n was performed, expressed as a percentage (%); and, (d) vigour

index, which determines the potential for a rapid germination/emergence, calculated as $(S_1/t_1)+(S_2/t_2)+ \ldots +(S_n/t_n)$.

2.4. Establishing an improved seed germination treatment

The main effects of the seven factors studied at two levels were evaluated using an L_8 (2^7) orthogonal array design (Roy, 2010) consisting of eight treatments (Table 1). These eight treatments are orthogonal and each of the two levels (L0 and L1) for each factor is represented in the different treatments the same number of times (four), of which for any factor one half (two) are evaluated at level L0, and the other half (two) are evaluated at level L1 for any other factor. For each treatment, six replicates (six Petri dishes, with 25 seeds per Petri dish) were used. Data on the four studied parameters were transformed using the arcsine transformation (inverse sine of the square root of percentage/100 for percentage data, and the proportion of the maximum possible value for the vigour index) and subjected to an ANOVA for testing the significance of differences of the treatments (Little and Hills, 1978). The significance of differences among the treatment means of transformed data was evaluated in transformed data using the Student-Newman-Keuls multiple range test at a P=0.05 (Hsu, 1996).

The degrees of freedom and sums of squares of the ANOVA for the eight treatments were partitioned in seven orthogonal contrasts for testing the significance of the main effect (i.e., the difference in the average between levels L0 and L1) for each factor (Little and Hills, 1978). Using this information, we proposed an improved protocol for the germination of *S. torvum* by including the level of each factor having a positive significant effect on the seed germination parameters studied.

2.5. Validation of the improved germination treatment

A first experiment was performed to evaluate if the orthogonal array method had been efficient in establishing an improved germination protocol under the experimental conditions (Petri dish germination) used for developing it. To validate the method used to establish the improved treatment we compared two treatments: (a) the proposed improved germination treatment according to the results of the orthogonal array experiment and (b) the best treatment out of the eight tested in the orthogonal array matrix. Six replicates (six Petri dishes, with 25 seeds per Petri dish) for each of these two treatments were used. Analyses and significance of differences of transformed data were performed by using an ANOVA as mentioned in section 2.4.

A second experiment was conducted to evaluate if the improved germination treatment obtained with the orthogonal array method under experimental conditions (germination in Petri dishes) was useful in improving the germination of *S. torvum* under commercial nursery conditions, by evaluating the emergence of seeds sown in a nursery growing substrate. In this experiment, the light factor had to be set at the level L0, as seeds were sown at a depth of 7 mm and germinated in the dark in all cases. The three treatments evaluated were a) *Solanum torvum* control treatment (all factors at level L0), b) the proposed improved germination treatment according to the results of the orthogonal array experiment, and c) *Solanum melongena* control treatment (all factors at level L0). Six replicates (six pots) for each of these three treatments were used. Data analyses and significance of differences were performed by using an ANOVA with transformed data as mentioned in section 2.4.

3. Results

3.1. Establishing an improved seed germination treatment

Highly significant differences ($P<0.0001$) were observed in the ANOVA analysis among the eight treatments (1 to 8) evaluated in the orthogonal array design for the four traits evaluated (Table

2). Treatments 5 and 6, which share levels L1 for soaking, L0 for NaClO and L1 for GA_3 (Table 1), had a high early germination, being significantly superior to the rest of the treatments (Figure 1). For treatments 2, 3, and 4 an early germination of some seeds was observed, but no significant differences were observed among them. The remaining treatments (1, 7, and 8) presented no germination at 4 d (Figure 1). The highest final germination at the end of the experiment (15 d) was found for treatments 2, 5 and 6 with average germination values above 99% and significantly higher values than the other treatments (Figure 1). Intermediate germination values were recorded for treatments 3, 4 and 8, with treatments 3 and 8 presenting significantly higher values than treatment 4. Finally, treatment 7 had a very low germination and treatment 1 no germination at all. Observation of the seeds sown in Petri dishes applying treatments 1 and 7 did not reveal any further germination even after 1 month.

The germination rate was highest (>98%) in treatments 5 and 6, which was significantly higher than that of the rest of the treatments (Figure 1). The next treatment with the highest germination rate was treatment 2, which was significantly higher than that of the treatments 3, 8 and 4. The lowest germination rate values were obtained for treatments 1 and 7 (Figure 1). The vigour index followed a similar pattern as the germination rate, with the highest values being those in treatments 5 and 6, and the lowest values coinciding with treatments 1 and 7. The significant groups for the vigour index were identical to those observed for the germination rate (Figure 1).

The orthogonal contrasts obtained from the partition of the degrees of freedom and sums of squares of the ANOVA for the treatments revealed that significant differences existed among the average values for the two levels (L0 and L1) for all factors in the four parameters studied, with the exception of the cold factor for the germination rate and vigour index (Table 2). The greatest F-ratios ($P<0.0001$) for early germination were obtained for the orthogonal contrasts of GA_3, NaClO and soaking. The remaining orthogonal contrasts were significant at $P<0.01$. For the final germination, all orthogonal contrasts were highly significant ($P<0.0001$) except for the cold factor ($P<0.01$), with the highest values being those for light, KNO_3, NaClO and GA_3. For the germination

rate, all the orthogonal contrasts were highly significant ($P<0.0001$), except for cold (Table 2). The highest F-ratio values were obtained for GA_3, light, KNO_3, and NaClO. For the vigour index, again cold was non-significant, and the remaining orthogonal contrasts were highly significant ($P<0.0001$), except for heat, which was significant at $P<0.01$ (Table 2).

The average values of level 1 (L1) were greater than those of level 0 (L0) for all factors across the four parameters studied with the exception of the NaClO factor, in which the values were greater for L0 (Table 3). For early germination, the highest L1-L0 absolute differences between levels were for GA_3, NaClO, and soaking, with values ≥40.0%. For the remaining factors these differences were <5% (Table 3). In the case of late germination and germination rate, the greatest differences between L1 and L0 were for the light, KNO_3, NaClO, and GA factors. Finally, for the vigour index, the greatest absolute differences were found for factors GA3, NaClO, soaking, and light (Table 3).

Based on the results obtained from the orthogonal contrasts for the primary effects of each factor tested and the average values for each of the levels of each factor, the following improved germination treatment is proposed for enhancing *S. torvum* seed germination: soaking: L1, NaClO: L0, GA_3: L1, KNO_3: L1, cold: L1, heat: L1, and light: L1 (Figure 1).

3.2. Validation of the improved germination treatment

The improved germination protocol proposed in section 3.1 was not among the treatments tested in the orthogonal array design. To validate the proposed treatment for its germination in Petri dishes, we compared it with treatment 6 of the orthogonal array design. Treatment 6, together with treatment 5, was significantly superior to the other treatments for all the parameters studied (except for the final germination of treatment 2, which did not differ significantly from treatments 5 and 6). Treatment 6 was chosen over treatment 5, as the former had a slightly higher (although non-significant) early germination (Figure 2). The improved treatment and treatment 6 differ in cold (L1

for the improved treatment and L0 for treatment 6) and light (L1 for the improved treatment and L0 for treatment 6) factors, with the remaining treatments applied at the same levels. No significant differences were obtained between the improved treatment and treatment 6 for any of the germination parameters studied (Table 4). The germination curves for both treatments are very similar, although values for the improved treatment are higher (although not significantly different) than those of treatment 6 (Figure 3). Germination occurred very quickly with more than 60% of the seeds germinated at 3 d and a germination plateau achieved at 6 d (Figure 3).

Regarding validation of the proposed method in the nursery growing substrate, highly significant differences ($P<0.0001$) were observed among the three treatments tested (*S. torvum* control, *S. torvum* improved treatment, and *S. melongena* control) for the seed emergence traits evaluated (Table 4). The seeds of *S. torvum* with the control treatment did not germinate (Table 5). *Solanum torvum* seeds with the improved treatment had approximately 50% early emergence (at day 7), which is significantly higher than that of the *S. melongena* control (Table 5). Conversely, the final germination of the control seeds of *S. melongena* was significantly higher than that of the *S. torvum* improved treatment. This resulted in a sharper sigmoidal curve in the *S. melongena* control compared to the *S. torvum* improved treatment (Figure 4). Regarding the emergence rate, no significant differences were observed between the *S. melongena* control and the *S. torvum* improved protocol (Table 5). However, for the vigour index, the values for the *S. torvum* improved treatment were significantly higher than those of the *S. melongena* control (Table 5). For both treatments, germination reached a plateau in 10 d.

4. Discussion

Although *S. torvum* is considered to be an outstanding rootstock for the commercial production of eggplant (Miceli et al., 2013; Moncada et al., 2014), its practical utilization is hampered by dormancy and poor germination (Ginoux and Laterrot, 1991; Miura et al., 1993;

308 Hayati et al., 2005). The efficient and successful production of high-quality grafted vegetable plants
309 requires an adequate synchronization of the development of rootstock and scion plantlets, which
310 requires the predictable germination of both the rootstock and scion (Lee et al., 2010). In this
311 respect, the germination protocol described here, which involves the application of a combination of
312 different factors having a positive effect on the germination of dormant seeds of *S. torvum*, has
313 proved highly efficient in producing a reliable, rapid and uniform germination in this species.

314 The use of an L8 orthogonal array experimental design allows the main effects on *S. torvum*
315 seed germination to be determined for seven factors using only eight treatments in which factors are
316 arranged in an orthogonal matrix (Roy, 2010). A few studies use orthogonal arrays for improving
317 seed germination in other species by studying only three (Wu et al., 2011) or four factors (Poinapen
318 et al., 2013). To the best of our knowledge, the present study is conducted with the largest number
319 of factors evaluated for improving seed germination. Our results show that similar to industrial and
320 biotechnological processes (Rao et al., 2008; Roy, 2010), orthogonal arrays are robust, powerful
321 and simple tools for simultaneously studying the primary effects of a large number of factors to
322 improve seed germination protocols in horticultural species. The primary advantages of orthogonal
323 arrays for seed germination testing is that they are much more efficient than studying one variable at
324 a time, and they are much simpler and less costly than full factorial designs (Little and Hills, 1978;
325 Onyiah, 2008; Rao et al., 2008).

326 All factors included in this study are known to have a potential effect on seed germination
327 (Finch-Savage and Leubner-Metzger, 2006; Bewley et al., 2013). In the present orthogonal array
328 experiment, we observed that all of the factors had an effect, although of varying aspects and
329 magnitude, on the seed germination of *S. torvum* seeds. The factors that exhibited a larger effect on
330 different seed germination parameters studied were soaking, NaClO, GA_3, KNO_3 and light. In all
331 treatments, except for NaClO, level L1 (application of the physical or chemical treatment) had a
332 positive effect compared to level L0 (no application of the treatment) on breaking the dormancy of
333 *S. torvum* seeds and improving early and final germination, as well as on the germination rate and

334 vigour index. In this respect, seed soaking for 12 to 24 h is known to be an efficient means for
335 improving the germination of *Solanum* species (Hayati et al., 2005; Ahmed et al., 2006), as it may
336 remove seed germination inhibitors (Bewley et al., 2013). Similarly, applications of the plant
337 growth regulator GA_3 or KNO_3 are efficient at releasing *Solanum* seeds from dormancy and
338 stimulating germination (Hayati et al., 2005; Wei et al., 2010; Gisbert et al., 2011a). Light
339 irradiation, which is an important regulator of seed germination in solanaceous species (Koo et al.,
340 2015), has also been observed to be efficient at stimulating germination in *S. torvum* seeds.
341 Amazingly, the scarification by NaClO had a highly negative effect on germination. NaClO
342 treatments are used for seed disinfection, but they also promote germination in some *Solanum*
343 species (Prohens et al., 1999). NaClO affects seed coat properties (Prohens et al., 1999) and this
344 may affect water uptake or other physical properties of the seed, in this case negatively, germination
345 (Bewley et al., 2013). We suggest that other suitable methods, other than NaClO treatement, for
346 seed disinfection and scarification should be used for *S. torvum*. Cold and heat factors also
347 influenced the germination of *S. torvum* such that the application of cold stratification and a heat
348 shock stimulated germination, although to a lesser extent than the other factors. In other studies,
349 cold or heat treatments proved efficient for releasing seeds of wild *Solanum* species from dormancy
350 (Shalimu et al., 2012; Koo et al., 2015). In this respect, cold induces the transcription GA_3 synthesis
351 genes (Penfield et al., 2005), whereas heat treatments result in the production of small heat-shock
352 proteins that stimulate germination (Koo et al., 2015).

353 Seeds of *S. torvum* presented strong physiological dormancy and did not germinate under
354 control conditions. This strong dormancy may be the underlying reason for the poor and irregular
355 germination problems of *S. torvum*, thus limiting its use as a rootstock (Ginoux and Laterrot, 1991;
356 Miura et al., 1993; Gousset et al., 2005; Hayati et al., 2005), as high and rapid germination was
357 recorded with some of the treatments tested in the orthogonal array design. Although some of the
358 treatments from the orthogonal array (e.g., treatments 5 and 6) provided excellent results with high
359 levels of early and final germination as well as a high germination rate, validation of the proposed

protocol is needed. The results of the comparison of the proposed improved method with the best treatment of the orthogonal array (treatment 6) confirmed the potential of the orthogonal array designs to determine an optimal combination of levels for each of the factors studied (Roy, 2010).

The evaluation of the improved protocol under conditions that simulate commercial nursery conditions (Lee et al., 2010) involved sowing the seeds in the growing substrate. Obviously, under these conditions the light irradiation treatment cannot be applied as seeds were covered by soil during germination. In this case, we also recorded that the control seeds of *S. torvum* did not germinate, confirming the strong dormancy in this species (Ginoux and Laterrot, 1991; Miura et al., 1993; Gousset et al., 2005; Hayati et al., 2005). However, a rapid germination in comparison to the non-treated *S. melongena* control, was obtained with the improved germination protocol. This result is important, as it indicates that this developed protocol may be applied commercially in the production of *S. torvum* plantlets as rootstocks for eggplant grafting. The slightly lower germination success compared to the Petri dish experiment may be caused by a lack of light irradiation, the different germination conditions, or both (Pensfield et al., 2005; Koo et al., 2015).

5. Conclusions

Fresh *S. torvum* seeds present a strong dormancy exhibiting no germination. The utilization of an orthogonal array design has been highly successful for estimating the main effects of factors affecting the seed germination of *S. torvum* seeds and for establishing an improved protocol for high and rapid germination. We determined that certain treatments, such as seed soaking, GA_3, KNO_3, and light irradiation, have highly positive effects in stimulating germination, whereas NaClO scarification causes negative effects. Cold scarification and heat shock also increased seed germination. The improved protocol results in a high and rapid germination under Petri dish and nursery growing substrate conditions. The results are of importance for the increased utilization of *S. torvum* as a rootstock for eggplant cultivation and for breeding programmes, and these findings

386 also demonstrate the utility of orthogonal arrays for establishing improved protocols for seed
387 germination involving many simultaneous factors.
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Table 1

L8 orthogonal array matrix (2^7) for the seven factors evaluated (soaking, NaClO, GA_3, KNO_3, cold, heat, and light) at two levels (L0 and L1), indicating the levels applied to each of the eight treatments tested.

	Factors							Day of
Treatment	Soaking	NaClO	GA_3	KNO_3	Cold	Heat	Light	initiation[a]
1	L0	L0	L0	L0	L0	L0	L0	0
2	L0	L0	L0	L1	L1	L1	L1	-8
3	L0	L1	L1	L0	L0	L1	L1	-2
4	L0	L1	L1	L1	L1	L0	L0	-8
5	L1	L0	L1	L0	L1	L0	L1	-9
6	L1	L0	L1	L1	L0	L1	L0	-3
7	L1	L1	L0	L0	L1	L1	L0	-9
8	L1	L1	L0	L1	L0	L0	L1	-1

[a] Beginning day of the application of the different levels so that the initiation (day 0) of the germination experiment is synchronized.

507 **Table 2**

508 Degrees of freedom, the F-ratio and its probability obtained from the ANOVA analyses for the effects of treatments and for the orthogonal

509 comparisons between the two levels for each factor tested on the *Solanum torvum* seed germination parameters.

Sources of variation	Degrees of freedom	Early germination (4 d; %)		Final germination (15 d; %)		Germination rate (%)		Vigour index	
		F-ratio	Prob. F	F-ratio	Prob. F	F-ratio	Prob. F	F-ratio	Prob. F
Treatments	7	157.5	<0.0001	238.0	<0.0001	228.8	<0.0001	235.8	<0.0001
Orthogonal contrasts									
Soaking	1	202.5	<0.0001	76.9	<0.0001	149.1	<0.0001	237.9	<0.0001
NaClO	1	402.2	<0.0001	318.3	<0.0001	271.0	<0.0001	329.2	<0.0001
GA_3	1	463.7	<0.0001	277.7	<0.0001	447.6	<0.0001	617.2	<0.0001
KNO_3	1	9.2	0.0043	369.3	<0.0001	272.9	<0.0001	175.3	<0.0001
Cold	1	8.2	0.0066	8.2	0.0067	0.3	0.6105	0.0	0.9362
Heat	1	8.8	0.0051	54.0	<0.0001	23.5	<0.0001	12.5	0.0011
Light	1	7.8	0.0079	561.8	<0.0001	437.4	<0.0001	278.7	<0.0001

510

511 **Table 3**

512 Average values of the *Solanum torvum* seed germination parameters for the two levels (level 0, L0; level 1, L1) of the different factors evaluated

513 and the differences between L1 and L0 (Δ L1-L0).

	Early germination (4 d; %)			Final germination (15 d; %)			Germination rate (%)			Vigour index		
Factors	L0	L1	Δ L1-L0[a]	L0	L1	Δ L1-L0	L0	L1	Δ L1-L0	L0	L1	Δ L1-L0
Soaking	8.2	48.2	40.0**	48.3	67.3	19.0**	42.6	65.0	22.4**	23.7	46.1	22.4**
NaClO	52.0	4.3	-47.7**	74.7	41.0	-33.7**	70.6	37.0	-33.6**	49.1	20.7	-28.4**
GA_3	3.8	52.5	48.7**	42.7	73.0	30.3**	36.6	71.0	34.4**	19.1	50.7	31.6**
KNO_3	26.3	30.0	3.7*	39.8	75.8	36.0**	38.5	69.2	30.7**	26.9	42.8	15.9**
Cold	26.5	29.8	3.3*	57.2	58.5	1.3*	53.7	53.9	0.2^{ns}	34.8	34.9	0.1^{ns}
Heat	26.0	30.3	4.3*	50.8	64.8	14.0**	48.0	59.6	11.6**	31.8	38.0	6.2*
Light	26.2	30.2	4.0*	33.5	82.2	48.7**	32.8	74.8	42.0**	23.9	45.9	22.0**

514 [a]**,*, ns indicate, respectively, significant at P<0.0001, P<0.01 or non-significant (see Table 2).

Table 4

F-ratio and its probability obtained from the ANOVA analyses for the seed germination parameters resulting from the comparisons between the two treatments for *Solanum torvum* seed germination in Petri dishes, and between the three treatments for *Solanum torvum* and *S. melongena* seed germination in a commercial nursery growing substrate.

	Petri dishes experiment[a]		Commercial substrate experiment[b]	
Parameter	F-ratio	Prob. F	F-ratio	Prob. F
Early germination	0.25	0.6309	48.26	<0.0001
Final germination	2.32	0.1586	424.37	<0.0001
Germination rate	2.06	0.1818	631.93	<0.0001
Vigour index	1.00	0.3407	551.07	<0.0001

[a] Germination treatments consisting of: (a) the optimal combination of factors of *S. torvum* (soaking: L1; NaClO: L0; GA_3: L1; KNO_3: L1; cold: L1; heat: L1; light: L1) according to the results obtained in the L8 orthogonal array design; (b) the best treatment of *S. torvum* (treatment 6; soaking: L1; NaClO: L0; GA_3: L1; KNO_3: L1; cold: L0; heat: L1; light: L0) out of the eight tested in the orthogonal array design matrix.

[b] Germination treatments consisting of: (a) the improved treatment of *S. torvum* consisting of the optimal combination of factors (soaking: L1; NaClO: L0; GA_3: L1; KNO_3: L1; cold: L1; heat: L1) according to the results obtained in the L8 orthogonal array design; (b) *S. torvum* control treatment (soaking: L0; NaClO: L0; GA_3: L0; KNO_3: L0; cold: L0; heat: L0); (c) *S. melongena* control treatment (soaking: L0; NaClO: L0; GA_3: L0; KNO_3: L0; cold: L0; heat: L0). The light factor was not tested as the seeds were covered by a 7-mm layer of substrate.

Table 5

Average values and comparison of means for the seed emergence parameters between the three treatments for *Solanum torvum* and *S. melongena* seed germination in a commercial nursery growing substrate.

Treatment[a]	Early emergence (7 d; %)[b]	Final emergence (14 d; %)	Emergence rate (%)	Vigour index
S. torvum control	0.0 a	0.0 a	0.0 a	0.0 a
S. torvum improved	49.3 c	77.3 b	60.8 b	52.6 c
S. melongena control	14.0 b	95.3 c	63.6 b	45.9 b

[a] *S. torvum* control = soaking: L0; NaClO: L0; GA_3: L0; KNO_3: L0; cold: L0; heat: L0. *S. torvum* improved = soaking: L1; NaClO: L0; GA_3: L1; KNO_3: L1; cold: L1; heat: L1. *S. melongena* control = soaking: L0; NaClO: L0; GA_3: L0; KNO_3: L0; cold: L0; heat: L0. The light factor was not tested as the seeds were covered by a 7-mm layer of substrate.

[b] Means separated by different letters are significantly different according to the Student-Newman-Keuls multiple range test at $P<0.05$.

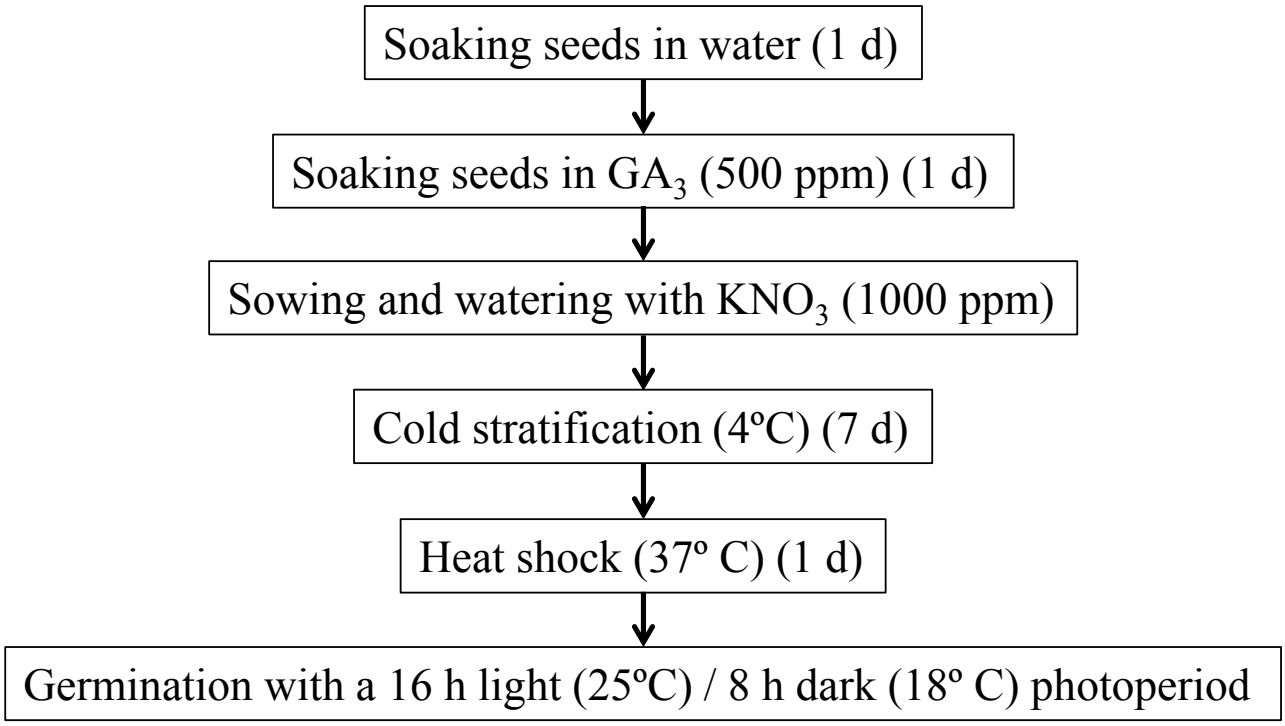


Fig. 1. Schematic representation of the improved protocol for enhancing *S. torvum* seed germination.

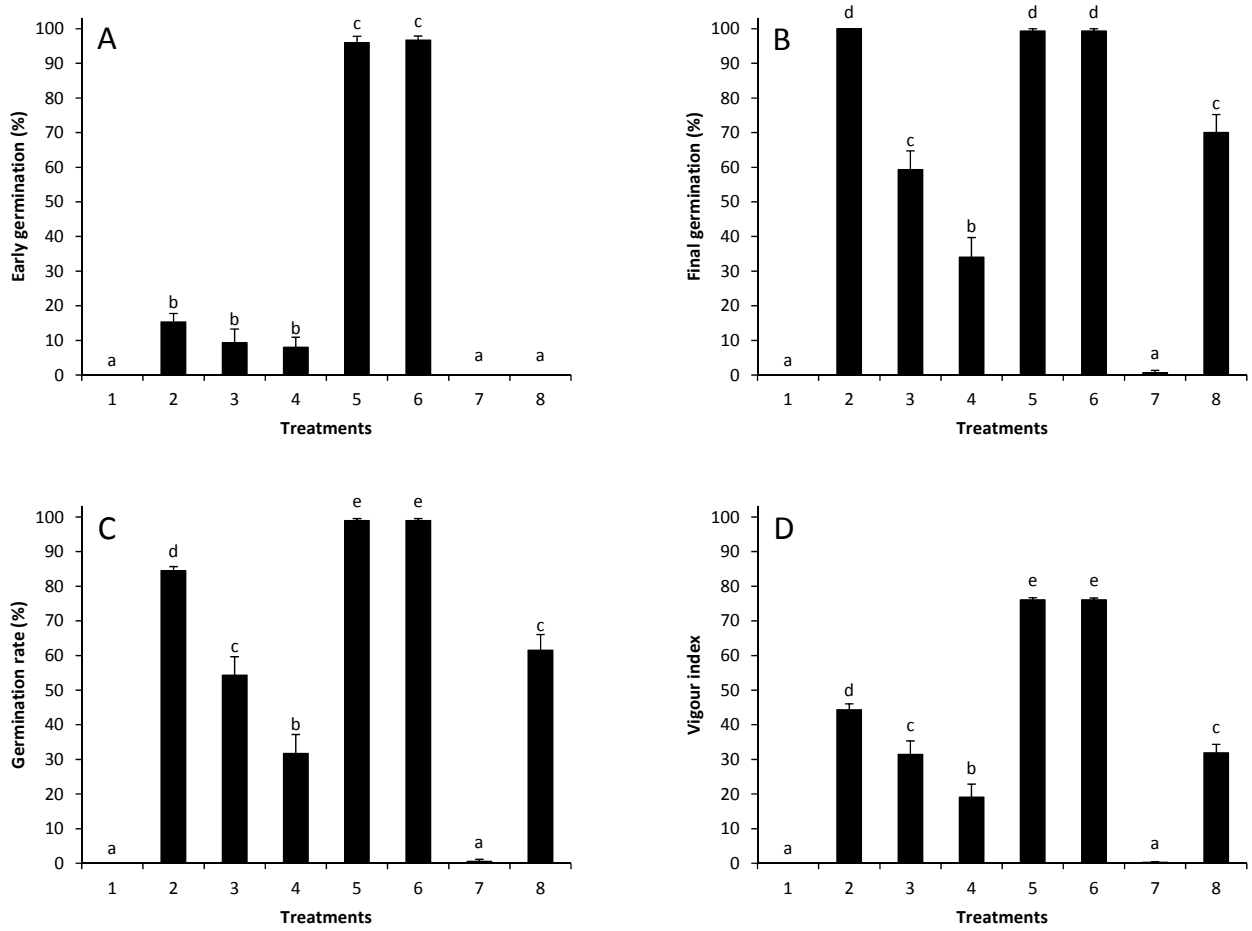
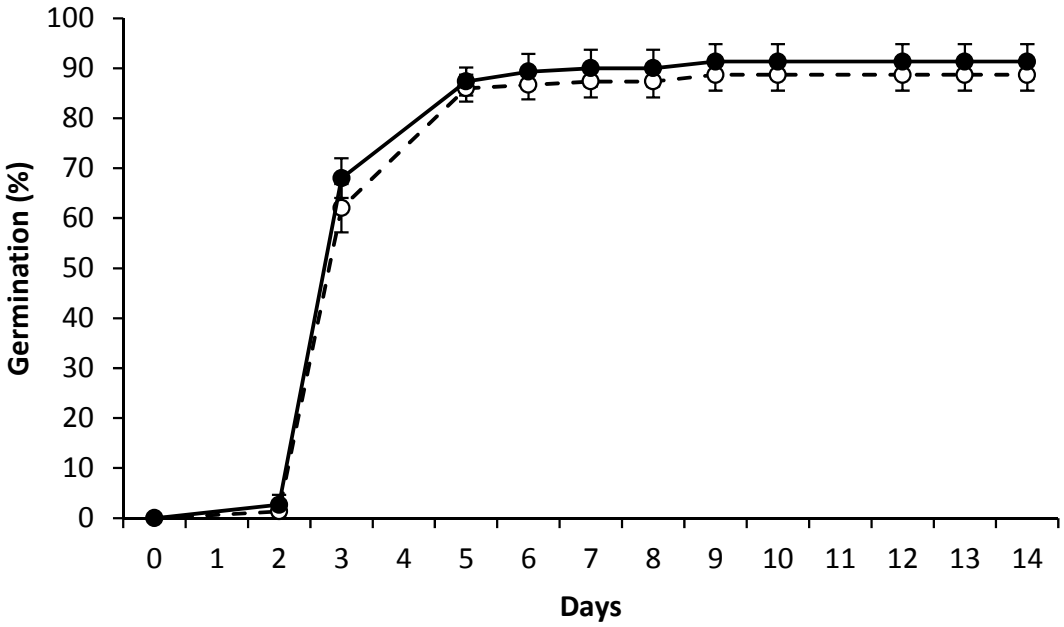


Fig. 2. Effect of the eight treatments tested in the L8 orthogonal array design on the four seed germination parameters: (A) early germination (4 d; upper left); (B) final germination (15 d; upper right); (C) germination rate (lower left); and, (D) vigour index (lower right). Bars represent the standard error (SE). Means separated by different letters are significantly different according to the Student-Newman-Keuls multiple range test at $P<0.05$.



551

552 **Fig. 3.** *Solanum torvum* germination curves for two treatments: a) the improved
553 treatment consisting of the optimal combination of factors (soaking = 1; NaClO = 0;
554 GA_3 = 1; KNO_3 = 1; cold = 1; heat = 1; light = 1) according to the results obtained in
555 the L8 orthogonal array design results (continuous line, black circles); and, b) the best
556 treatment (treatment 6; soaking = 1; NaClO = 0; GA_3 = 1; KNO_3 = 1; cold = 0; heat = 1;
557 light = 0) out of the eight tested in the orthogonal array design matrix (dashed line,
558 white circles). Bars represent the standard error (SE).

559

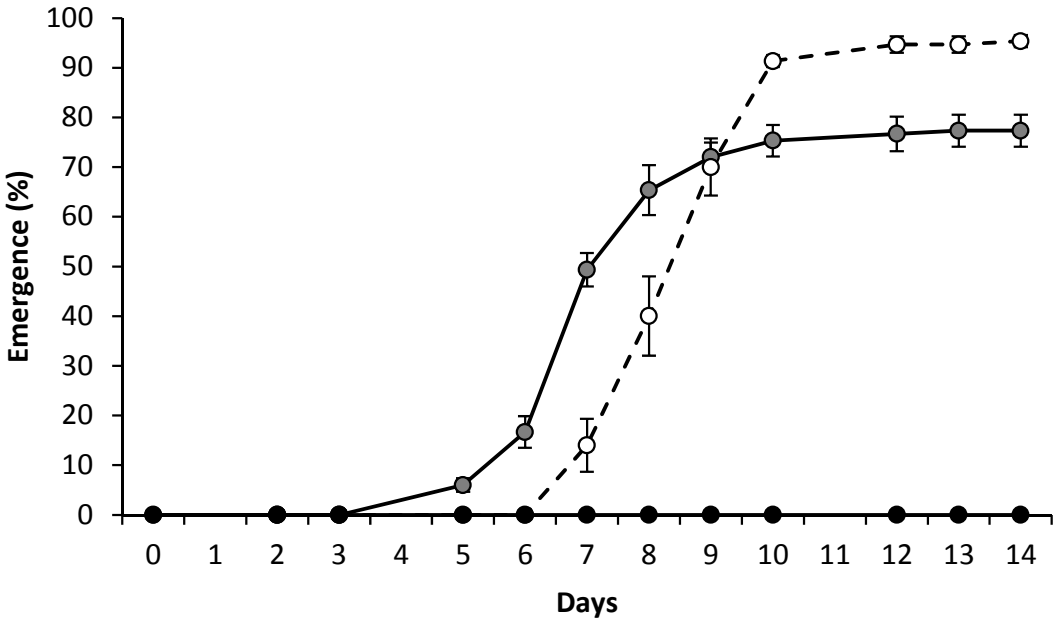


Fig. 4. Emergence curves for *Solanum torvum* and *S. melongena* sown in a commercial nursery growing substrate for three treatments: a) *S. torvum* control treatment (soaking = 0; NaClO = 0; GA_3 = 0; KNO_3 = 0; cold = 0; heat = 0) (continuous line, black circles); b) *S. torvum* improved treatment consisting of the optimal combination of factors (soaking = 1; NaClO = 0; GA_3 = 1; KNO_3 = 1; cold = 1; heat = 1) according to the results obtained in the L8 orthogonal array design (continuous line, grey circles); and, c) *S. melongena* control treatment (soaking = 0; NaClO = 0; GA_3 = 0; KNO_3 = 0; cold = 0; heat = 0) (dashed line, white circles). The light factor was not tested as the seeds were covered by a 7-mm layer of substrate. Bars represent the standard error (SE).