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Ahmed M. Abbas
King Khalid University

Mohamed Abdelazeem
South Valley University

Stephen J. Novak
Boise State University

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Ahmed M. Abbas [1,2,*], Mohamed Abdelazeem [2] and Stephen J. Novak [3,*]

[1] Department of Biology, College of Science, King Khalid University, Abha 61413, Saudi Arabia
[2] Department of Botany and Microbiology, Faculty of Science, South Valley University, Qena 83523, Egypt
[3] Department of Biological Sciences, Boise State University, Boise, ID 83725, USA
* Correspondence: ahhassan@kku.edu.sa (A.M.A.); snovak@boisestate.edu (S.J.N.); Tel.: +96-65-4072-1385 (A.M.A.); +1-208-426-3548 (S.J.N.)

Abstract: Anaerobic digestion (AD) involves the breakdown of a substrate by mixed microbial communities in the absence of free oxygen. This process has many benefits leading to the production of bioenergy (biogas) and fertilizers (bio-fertilizers). Unfortunately, the bio-fertilizer made using AD may be contaminated with weed seeds and may have the potential of infesting the fields to which it is applied. Thus, the goal of this study is to determine the effects of AD on seed germination and viability of two undesirable crop plants (*Triticum aestivum* and *Sorghum bicolor*) and four weed species of reclaimed agricultural land near Qena, Egypt (*Schouwia purpurea*, *Polygonum equisetiforme*, *Amaranthus viridis*, and *Lotus arabicus*). Vegetative biomass of *T. aestivum* was used as the substrate, and cattle rumen fluid was used as the inoculum. The seeds of the six plants were placed in culture bottles and subjected to AD at 37 ± 2 °C for up to 16 days. Time to first germination (the time required for the first seed to germinate) and the percentage of seeds that germinated varied among the six species. The percentage germination of all six species decreased with increasing duration of AD, with no seeds germinating at 12 or 16 days of incubation. Seeds of *T. aestivum* and *S. bicolor* experienced a rapid and significant decrease in the percentage of viable seeds and an increase in the percentage of non-viable seeds. In contrast, even though they did not germinate, some seeds of the other four weed species remained viable, even after 16 days of incubation. Approximately 32% of *S. purpurea* seeds remained viable at 16 days of incubation. Overall, our results suggested that the application of anaerobic digestate in the reclaimed agricultural lands near Qena, Egypt, is not likely to exacerbate weed infestations in these fields.

Keywords: bio-fertilizer; reclaimed agricultural lands; weed infestations; anaerobic digestate; *Amaranthus viridis*; *Lotus arabicus*; *Polygonum equisetiforme*; *Schouwia purpurea*; *Sorghum bicolor*; *Triticum aestivum*

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1. Introduction

Anaerobic digestion (AD) involves the breakdown of substrates (organic matter) into a soluble form by mixed microbial communities (also known as inoculum) in the absence of free oxygen [1,2]. The anaerobic digestion of various substrates, including animal manure, other organic waste, and crop plant residue, is a method of managing agronomic wastes because it reduces the amount of organic matter that requires disposal. In addition, AD can be used to meet global energy needs because it leads to the production of bioenergy (biogas) through the generation of methane [3–10]. Biogas production represents a well-established, environmentally friendly (green), renewable, and sustainable alternative to burning fossil fuels [1,2,4,9,10]. In 2004, burning fossil fuels accounted for 80% of the Earth's energy consumption [11], which increased the concentration of atmospheric CO_2 and other greenhouse gases [8] and has contributed to climate change and extreme weather events [12,13]. Biogas production by AD substantially reduces the production of CO_2 and other greenhouse gases [4,9], thereby reducing the increase in the concentration of atmospheric CO_2

currently taking place. In addition to reducing organic waste and providing bioenergy production, AD can lead to reductions in the release of human pathogens, other microorganisms, and organic micropollutants into the environment. Anaerobic digestion also reduces the uncontrolled release of excessive amounts of nutrients into the environment, both onto land and into bodies of water [1,4,7].

Anaerobic digestate (referred to as digestate or sludge) is a semi-solid material generated from the AD of animal manure, organic waste, and crop plant residue [1,4–6,9]. Digestate is made up of microbial biomass, partially degraded organic matter, and inorganic compounds and can be used as (1) a source of nutrient-rich, slow-release bio-fertilizer) for conventional and organic agricultural crops, (2) as an inorganic substitute fertilizer, and (3) as a raw material for bio-fertilizer production [14,15]. The digestate is an eco-friendly alternative to chemical fertilizers because its application to agronomic systems has additional benefits: it supplies the nutrients required for increased crop growth and higher reproductive output (crop yield), it reduces the loss of organic matter from agricultural soils, and it stimulates the metabolism of soil microbes and soil enzymes [14]. In addition, the use of digestate as a bio-fertilizer has advantages over the use of animal manure or an animal slurry because it has a lower C/N ratio, a higher concentration of K, P, and N, increased pH, improved fluidity, and possesses less odor than both manure and animal slurries [1,5,6].

Despite the benefits of digestate as a bio-fertilizer, the use of digestate also has some disadvantages. Digestate with higher amounts of mineral N (ammonia) and lower amounts of organic C, compared to the C/N ratio of undigested manure, may be a concern when used in certain agronomic systems (e.g., organic farms) [7,16]. These higher amounts of ammonia can rapidly be nitrified to nitrate and lost through denitrification [7]. Digestate with high amounts of ammonia may also result in phytotoxicity, increased soil salinity, increased nutrient runoff into bodies of water, and can be leached to groundwater [17–20]. Digestate bio-fertilizers may also contain various nutrients and heavy metals, which may adversely affect the bioavailability of elements in the soil of agricultural fields [17,18]. The impacts of digestate application on soil processes are still poorly understood [18], and additional research on this issue is warranted.

Of major concern is the fact that the various substrates used in biogas and bio-fertilizer production (animal manure, organic waste, and crop plant residue) may be contaminated by animal intestinal parasites and pathogens, human pathogens, plant pathogens, and plant propagules and seeds [1,4–7,9,10]. If these pathogens and plant seeds survive the AD process and are released into the environment from the bio-fertilizer, they may proliferate and cause livestock parasitism and disease, human diseases, plant diseases, and weed infestations in the agronomic systems into which the bio-fertilizer is applied [1,7,21]. Spore-forming bacteria are more resistant to anaerobic digestion than fungi and plant seeds, while fungi and seeds exhibit similar abilities to resist AD [1]. Many factors contribute to the plant seed contamination in the digestate produced during the AD process and include the choice of components used to perform AD [i.e., which substrates and which inoculum or microbial communities (digested manure, septic waste, or animal rumen) are used], the abundance of plant seeds in the substrate used in the digestion process, the duration, operational temperature, and pH of the AD conditions, whether these seeds have high seed coat hardness or other protective structures, and the hydration level, metabolic rate, and dormancy mechanisms of the plants [1,4–7,9,10]. In general, seeds can endure and survive very harsh conditions because the seed stage is the most resistant portion of the plant life cycle. Moreover, plant seeds vary considerably in their characteristics and properties and therefore have been shown to differ in their ability to survive and germinate following AD [1].

Egypt's total land area is 1 million km^2; almost 96% of this area consists of vast desert tracts, and only 4% of Egypt's territory is under cultivation [22–24]. In addition, Egypt has one of the world's fastest-growing populations, with an estimated population size of 100 million people in 2019 [23]. Consequently, a portion of Egypt's population is at risk of

food insecurity. The three major agricultural regions in Egypt are oases (e.g., the Fayium Oasis), the Nile River Delta, and the Nile River Valley. In addition, over many decades, the Egyptian government, local governorates (e.g., the Qena Governorate), private companies, and farmers have engaged in extensive efforts to expand the amount of cultivated land through desert reclamation [22–25], which have increased the area cultivated in Egypt by more than 1.2 million hectares (12,000 km^2) in the last 50 years [22]. These efforts have targeted barren areas in the Nile River Delta and barren areas in the Eastern Desert and the Western Desert adjacent to the Nile River Valley for reclamation [23] (Figure 1).

Figure 1. Photos showing two reclaimed agricultural fields near Qena, Upper Nile Valley, Egypt.

The reclamation and sustainable use of these former desert lands are contingent on two factors: the availability of surface and groundwater resources for reclamation and irrigation [26], and the application of fertilizers to these croplands, especially local sources of organic bio-fertilizers [27,28]. Thus, there are increasing efforts in Egypt to employ AD to convert agricultural wastes into bio-fertilizer (and biofuel) [27]. However, as outlined above, there is also an awareness of how these efforts may worsen weed infestations (and other problems) within agricultural fields and spread weeds to new fields.

Across Egypt, El Hadid et al. [29] indicated that approximately 470 weed species occur (constituting about 22.5% of the flowering plants in Egypt), with 185 of these species being weeds of cultivated farmlands. The weed flora of reclaimed desert lands in various regions in Egypt have been described [30,31], and these studies reported relatively high levels of weed species diversity because these human-created habitats are environmentally heterogeneous, experience frequent and varied disturbance events, and are colonized by non-native plant species [30]. The weed flora of common crops in desert reclaimed lands of the Qena Governorate was assessed and revealed a total of 169 vascular plant species in these fields [31], which belong to 121 genera in 39 families, with the most species-rich families being Poaceae, Asteraceae, Fabaceae, Brassicaceae, Chenopodiaceae, and Euphorbiaceae. These 169 plant species were assigned to five different rarity categories based on the size of their range, habitat specificity, and abundance: dominant species, very common species, common species, occasional species, and sporadic species [31].

Extensive efforts to expand the amount of cultivated land in Egypt through desert reclamation will continue in the future. Moreover, these reclaimed lands will require fertilizer inputs, and the use of bio-fertilizer produced through AD has been proposed [27]. Thus, there is a need to determine whether the use of digestate in reclaimed lands will contribute to the introduction and spread of plant species in the agronomic systems into which the digestate has been applied. The goal of this study is to determine the effects of AD on the germination and viability of the seeds of six common plant species of reclaimed agricultural land near Qena, Egypt. This study represents an initial assessment of the risk of applying digestate contaminated with plant seeds to these reclaimed lands.

2. Materials and Methods

2.1. Study Area and Weed Seed Selection

The study area is in the Upper Nile Valley near Qena, Egypt (26°10′32.82″ N–32°38′57.76″ E), and it is bordered to the east by the Eastern Desert and to the west by the Western Desert [31,32]. Near Qena, the Nile River broadens and makes a sharp bend. Cultivated lands are located between the river and limestone cliffs that reach an elevation of 300 m, with older cultivated lands (cultivated since ancient times) bordering the river. Recently reclaimed cultivated lands (in use for approximately 30 years) are located between the older cultivated lands and the border of the two deserts [31]. The Eastern and Western Deserts of Egypt are parts of the Sahara Desert of North Africa; therefore, summers in Qena are long and arid and can last for about five months, with an average high temperature in July of 40 °C. The cool season lasts for approximately three months, with the coolest month of the year being January (average low temperature is 8 °C) [31,32]. Rainfall is negligible and does not usually exceed 0.55 mm/year [32]. Thus, irrigation of agricultural crops near Qena, using surface and groundwater, is necessary. Reclaimed agriculture lands in the region near Qena were visited to identify candidate plant species in these fields. The goal was to select plants representing the five rarity categories established by Salama et al. (2016) [31]. In addition, plants were selected that exhibited a range of seed coat hardness and dormancy requirements. We collected seeds from six plant species for this study (Table 1): *Triticum aestivum* L. (Poaceae; bread wheat), *Sorghum bicolor* (L.) Moench (Poaceae; sorghum), *Schouwia purpurea* (Forssk.) Schweinf. (Brassicaceae, schouwia), *Polygonum equisetiforme* Sibth. & Sm. (Polygonaceae; horsetail knotweed), *Amaranthus viridis* L. (Amaranthaceae; slender amaranth), and *Lotus arabicus* Sol. ex L. (Fabaceae; trefoil). *Triticum aestivum* and *S. bicolor* are undesirable crop plants that grow in *Medicago sativa* L. (alfalfa) fields in our study area [32]. Salama et al. [31] classified *P. equisetiforme* as a "dominant" plant (weed) species in the Qena region, *S. bicolor* and *S. purpurea* were classified as "common" plant species, *T. aestivum* and *A. viridis* were classified as "occasional" plant species, and *L. arabicus* was not classified. *Triticum aestivum* and *S. bicolor* have low seed coat hardness and dormancy requirements, whereas members of Fabaceae (e.g., *L. arabicus*) typically possess high seed coat hardness (their seed coats have a water-impermeable layer), high dormancy, and low rates of germination even under favorable conditions.

Table 1. Time to first germination (in days) for the six weed species, across six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). Different letters indicate time to first germination values that are significantly different ($p < 0.05$).

Species	Time to First Germination (Day)						
	Control	1	2	4	8	12	16
Triticum aestivum	1 ± 0^{b}	2 ± 0^{d}	5 ± 0^{c}	5 ± 1^{c}	9 ± 0^{a}	0 ± 0	0 ± 0
Sorghum bicolor	1 ± 0^{b}	4 ± 0^{c}	8 ± 1^{b}	7 ± 1^{b}	9 ± 0^{a}	0 ± 0	0 ± 0
Schouwia purpurea	1 ± 0^{b}	2 ± 0^{d}	3 ± 1^{d}	7 ± 0^{b}	5 ± 2^{b}	0 ± 0	0 ± 0
Polygonum equisetiforme	2 ± 0^{a}	17 ± 0^{a}	17 ± 3^{a}	0 ± 0^{d}	0 ± 0^{c}	0 ± 0	0 ± 0
Amaranthus viridis	2 ± 0^{a}	2 ± 0^{d}	4 ± 0^{d}	8 ± 1^{b}	8 ± 1^{a}	0 ± 0	0 ± 0
Lotus arabicus	2 ± 0^{a}	10 ± 0^{b}	0 ± 0^{e}	20 ± 0^{a}	0 ± 0^{c}	0 ± 0	0 ± 0

2.2. Substrate and Inoculum

Anaerobic digestion was generally conducted according to the methods of Eckford et al. [4], using batch cultures (reactors) with a substrate consisting of 5% total solids. Vegetative biomass of *T. aestivum*, which is a common agricultural crop in the Qena region, was used as the substrate, and cattle rumen fluid obtained at a local slaughterhouse was used as the inoculum. This rumen fluid was chosen because it contains a complex bacterial community capable of living under strictly anaerobic conditions [33–35]. Additionally, the microorganisms in the rumen inoculum produce and excrete specialized enzymes that have hydrolytic activity capable of digesting the lignocellulose of *T. aestivum* biomass [35,36].

We conducted AD using Wheaton^{TM} culture bottles, and each bottle was partially filled with inoculum and an appropriate amount of wheat substrate to achieve a $VS_{inoculum}/VS_{substrate}$ ratio of 2; this ratio minimizes diffusion limitation and avoids acidification or toxicity inhibition [37]). To ensure that AD occurred, reactor contents were thoroughly mixed and initially incubated at 55 °C for 24 h in a C24 incubation shaker (New Brunswick Scientific Company, Edison, NJ, USA). Lights were excluded from the culture bottles to simulate the digestion conditions of an animal's rumen. Four other reactor bottles with the same components, but no seeds, were monitored for biogas production and quality using the liquid displacement method, according to Kamel et al. [38].

2.3. Anaerobic Digestion Treatments

After the initial 24-h incubation period described above, the culture bottles were opened, and nylon mesh bags containing 50 seeds of the six plant species were placed into the culture fluid. The meshed bags allowed seeds to be immersed in the culture fluid while also ensuring that seeds remained within the bags. The bottles were then sealed and returned to the incubator. The total volume in the culture fluid was 500 mL, and the culture temperatures ranged from approximately 30 to 40 °C. Two to three hours after the seeds were placed in the culture bottles, the bottles were analyzed to ensure that anaerobic digestion had resumed, using the method described above [38]. For all treatments, i.e., the six plant species across all six incubation time intervals, three replicates (three culture bottles) were established. Seeds in the AD culture bottles were allowed to incubate for six time periods: 1, 2, 4, 8, 12, and 16 days. These incubation time periods (retention times) were chosen because they are like the time intervals used in similar studies conducted by Johansen et al. [7] and Zhou et al. [10]. At these designated time periods, the nylon bags containing seeds from each of the six plant species were removed from the culture bottles, and the seeds were prepared for germination and viability tests. Data for the controls reported here, for the seeds of each of the six plant species, are for untreated seeds that were never incubated in the AD culture bottles.

2.4. Seed Germination and Viability

At each incubation time interval, seeds of each of the six weed species were removed from the nylon bags, and the seeds were disinfected by immersion in a 1% sodium hypochlorite solution for 2 min and then thoroughly rinsed with sterile distilled water for 10 min [39]. Fifteen seeds from each bag were randomly selected, and the seeds were placed in a 9-cm Petri dish containing filter paper (we set up 3–5 Petri dishes per treatment), and 5 mL of distilled water was added to each dish. Petri dishes were then wrapped with parafilm and placed in a germinator (Lab Line Instruments Inc., Melrose Park, IL, USA) for up to 20 days at 20 °C under a 12/12-h light/dark regime. This temperature was chosen because it is the optimal temperature for seed germination for the six plant species we analyzed [40,41]. Dishes were inspected daily, and the seeds that germinated were recorded. A seed was considered to have germinated when its radicle emerged [42]. Time of first germination (the time required for the first seed to germinate), total germination percentage, percent of viable seeds, and percent of non-viable seeds were calculated [42,43]. Seed viability of the remaining ungerminated seeds was determined using the tetrazolium test, as described by Mancilla-Leyton et al. [44]. The value of the percent germinated seeds, percent viable seeds, and percent non-viable seeds sums to 100%.

2.5. Anaerobic Digestion Culture Conditions

Anaerobic digestion culture conditions, which can influence the survival of seeds during incubation, were measured at each incubation time interval using four parameters: temperature, electrical conductivity (EC), total dissolved solids (TDS), and pH. Values for the controls were measured just after the anaerobic culture bottles were set up, without the addition of plant seeds or incubation. After the nylon bags containing seeds were removed from the culture bottles, at each incubation time interval, the temperature of

the digestate was immediately determined, and a 1:5 aqueous extract of digestate to culture fluid (1:5 ratio on a dry weight basis) was obtained and used to measure EC and pH [45]. Electrical conductivity was determined using an EC meter (EC Meter 307, Canfort Laboratory and Educational Supplies Co., Guangzhou, China), and pH was measured using a pH meter (PHSJ-3F pH Meter, Labo-Hub, Shanghai, China) [45,46]. Values of TDS were calculated using EC values according to the method described by Rudydi [47], using the following equation, TDS (mg/L) = $k_e \times$ EC (μS/cm), where k_e is a constant that ranges from 0.5 to 0.8, with a value of 0.60 most often recommended for use [47].

2.6. Statistical Analysis

Data generated in this study were tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variance using Levine's test. Variation in the data was calculated as the standard error (SE) of the mean. Statistically significant differences in the number of days to first germination, final germination percentage, percent of viable seeds, percent non-viable seeds, and AD conditions (temperature, ED, TDS, and pH) for the six weed species at the six time periods were tested using one-way analysis of variance (ANOVA) (*F*-test). Tukey's honestly significant difference (HSD) test was used to determine the significance among means for treatments with a significant *F*-test [39,42,43]. A significance level of $p < 0.05$ was applied for all analyses. SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

3.1. Seed Germination and Viability

The six weed species showed highly significant statistical differences for almost all the seed germination and viability parameters across all the time periods we assessed in this study, except for the percent of viable seeds at 12 days of incubation (see below). In addition, statistically significant differences for time to first germination and percent of seeds that germinated at 12 and 16 days of incubation could not be determined because no seeds germinated at these two time periods.

Time to first germination (the time required for the first seed to germinate) varied among the six weed species we tested. For controls (seeds never incubated in the anaerobic digestion (AD) culture bottles), at least one seed of *T. aestivum*, *S. bicolor*, and *S. purpurea* germinated in one day, whereas seeds of *P. euisetiforme*, *A. veridis*, and *L. arabicus* required two days to first germination (Table 1). Differences among the six species were statistically significant (Table S1). In addition, values for time to first germination for all six weed species increased over the course of the experiment, i.e., with increased time of incubation in the AD conditions, time to first germination increased. For instance, at 4 days of incubation, at least one seed of *L. arabicus* required 20 days to germinate, while the time to first germination of *T. aestivum* at 8 days of incubation took 9 days (Table 1). Differences in time to first germination among the six weed species were statistically significant over the first four time periods (Table S1). For all six plant species, no seeds germinated after 12 or 16 days of incubation.

All six plants exhibited variation in the percentage of seeds that germinated [Germination (%)]. For the controls, *T. aestivum* and *S. bicolor* had the highest percent germination (at or near 100%), *P. euisetiforme* had 86.7% germination, *S. purpurea* had 73.3% germination, *A. veridis* had 60.0% germination, and *L. arabicus* had 10.0% germination (Figure 2, Table S2). Differences among controls for the six species were statistically significant (Table S1). The percentage germination of all six species decreased with increasing duration of AD, with no seeds germinating at 12 or 16 days of incubation. Differences in percent germination among the six plant species were statistically significant over the first four time periods (Table S1). *Amaranthus viridis* had the least reduction in percent germination, with 35.2% of seeds germinating after 8 days of incubation. Over the incubation times used in this study, *T. aestivum*, *S. bicolor*, and *S. purpurea* generally had similar values for percent germination.

Of the six weed species, *P. equisetiforme* and *L. arabicus* had the lowest values for percent germination (Figure 2, Table S2).

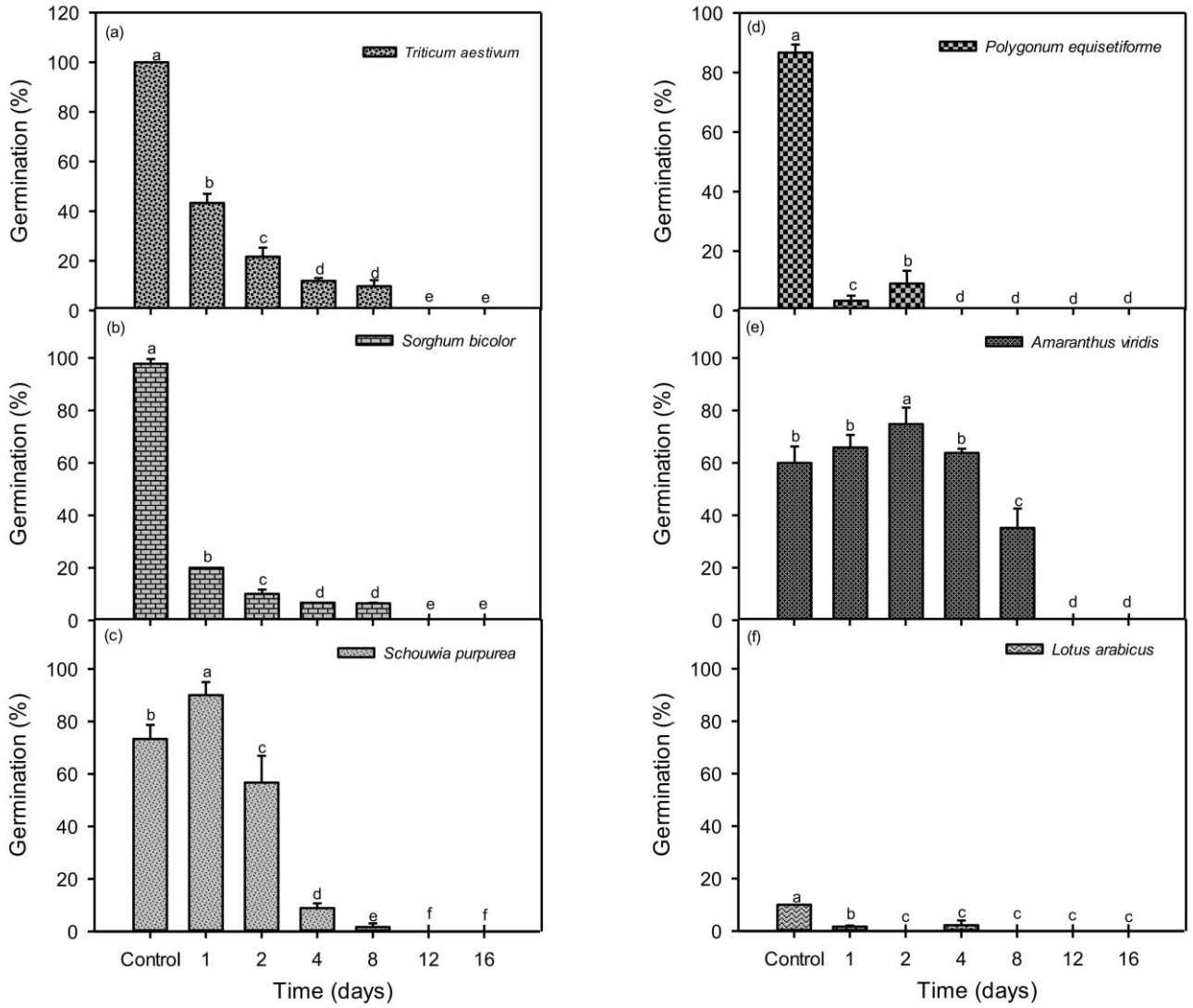

Figure 2. Percentage of the seeds of the six weed species that germinated, across six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). The six panels present the data for each of the study species: (**a**) *Triticum aestivum*, (**b**) *Sorghum bicolor*, (**c**) *Schouwia purpurea*, (**d**) *Polygonum equisetiforme*, (**e**) *Amaranthus viridis*, and (**f**) *Lotus arabicus*. Different letters at the top of the histograms indicate percent germination values that are significantly different ($p < 0.05$).

Over the incubation times used in this study, seeds of the six species exhibited statistically significant differences in the percent of viable seeds, except for 12 days of incubation (Table S1). The percent viable seeds and percent non-viable seeds for all six plant species for the control treatments and across six AD incubation time periods are presented in Table 2. *Triticum aestivum* and *S. bicolor* exhibited rapid and significant decreases in the percentage of viable seeds and an increase in the percentage of non-viable seeds (Table 2). These results suggest that the seeds of these two species are not capable of surviving the anaerobic digestion conditions imposed in this study. In contrast, even though they did not germinate, some seeds of the other four plants remained viable, even after 16 days of incubation. Seeds of *S. purpurea* retained the highest amount of viability at 16 days of incubation (32.5%), whereas seeds of *P. equisetiforme*, *A. viridis*, and *L. arabicus* had lower, and similar values of seed viability, 6.7%, 8.8%, and 9.7%, respectively (Table 2). Even though some seeds of the latter three species did retain low levels of viability, the percentage of non-viable seeds for

them is approximately 90.0%. Differences in the percent of non-viable seeds among the six plant species were statistically significant for the controls and across all six incubation time periods (Table S1).

Table 2. Percent viable and percent non-viable seeds for the six plant species, across six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). Different letters indicate percent viable and non-viable seed values that are significantly different ($p < 0.05$).

	Species					
Incubation Time (day)	***Triticum aestivum***		***Sorghum bicolor***		***Schouwia purpurea***	
	Viable (%)	**Non-viable (%)**	**Viable (%)**	**Non-viable (%)**	**Viable (%)**	**Non-viable (%)**
Control	0 ± 0^{d}	0 ± 0^{f}	2.2 ± 1.8^{d}	0 ± 0^{f}	28.9 ± 2.1^{d}	2.2 ± 1.8^{e}
1	50.0 ± 3.7^{a}	6.7 ± 0.1^{e}	66.7 ± 2.6^{a}	13.3 ± 2.4^{e}	1.7 ± 1.4^{e}	8.3 ± 4.3^{d}
2	48.3 ± 3.6^{a}	30 ± 3.7^{d}	64 ± 1.4^{a}	27 ± 2.8^{d}	36.6 ± 7.7^{b}	6.7 ± 1.1^{d}
4	41.6 ± 2.7^{b}	46.5 ± 2.4^{c}	53.3 ± 2.3^{b}	40 ± 2.1^{c}	51.2 ± 1.8^{a}	39.9 ± 1.7^{c}
8	42.7 ± 3.4^{b}	47.5 ± 1.8^{c}	19.3 ± 1.3^{c}	74.3 ± 2.7^{b}	37.1 ± 4.6^{b}	61.2 ± 4.4^{b}
12	10.8 ± 1.4^{c}	89.2 ± 6.3^{b}	4.3 ± 1.8^{d}	95.7 ± 3.8^{a}	39.8 ± 2.7^{b}	60.2 ± 3.6^{b}
16	0 ± 0^{d}	100 ± 0^{a}	0 ± 0^{e}	100 ± 0^{a}	32.5 ± 0.1^{c}	67.5 ± 0.6^{a}
	Species					
Incubation Time (day)	***Polygonum equisetiforme***		***Amaranthus viridis***		***Lotus arabicus***	
	Viable (%)	**Non-viable (%)**	**Viable (%)**	**Non-viable (%)**	**Viable (%)**	**Non-viable (%)**
Control	6.7 ± 0.5^{e}	6.6 ± 1.2^{e}	$22.2 \pm 1.6^{a,b}$	17.8 ± 1.2^{e}	84.4 ± 1.8^{a}	5.6 ± 0.3^{f}
1	93.3 ± 3.1^{a}	3.4 ± 0.02^{f}	25.4 ± 1.4^{a}	7.5 ± 0.3^{f}	$74.5 \pm 2.8^{a,b}$	23.8 ± 1.3^{e}
2	76.5 ± 2.2^{b}	14.4 ± 2.4^{d}	5 ± 1.4^{d}	21.2 ± 5.4^{d}	38.3 ± 4.9^{d}	61.7 ± 3.2^{b}
4	24.1 ± 0.3^{c}	75.9 ± 1.4^{c}	8.4 ± 0.3^{c}	27.8 ± 2.3^{d}	60 ± 1.8^{c}	37.8 ± 1.3^{c}
8	18.2 ± 3.9^{d}	82.8 ± 3.3^{b}	18.5 ± 1.3^{b}	46.3 ± 1.1^{c}	70 ± 5.4^{b}	30 ± 2.1^{d}
12	1.45 ± 0.1^{f}	98.5 ± 1.5^{a}	17.5 ± 0.4^{b}	82.5 ± 1.3^{b}	15.9 ± 1.3^{e}	84.1 ± 3.6^{a}
16	6.7 ± 0.8^{e}	93.3 ± 0.7^{a}	8.8 ± 2.19^{c}	89.2 ± 1.7^{a}	9.7 ± 4.1^{f}	90.3 ± 5.2^{a}

3.2. Anaerobic Digestion Culture Conditions

Culture conditions during AD can influence the survival of seeds during incubation. All four culture condition parameters measured here exhibited statistically significant differences across all the time periods assessed in this study. Temperatures for the six AD incubation time periods differed significantly and increased from 28.6 °C at 1 day of incubation to 39.8 °C at 16 days of incubation (Table 3). Electrical conductance (EC) and TDS values increased steadily and significantly over the course of the incubation period (Table S3), with the highest values of both parameters occurring after 12 and 16 days of incubation (Figure 3). pH values were significantly different (Table S3), and peaked at 2 days of incubation, with a value of approximately 8.0. These values decreased over the next intervals until pH values stabilized at approximately 7.0 at 8, 12, and 16 days of incubation (Figure 3).

Table 3. Temperatures for the six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). Different letters indicate temperature that are significantly different ($p < 0.05$).

Incubation Time (Day)	Temperature (°C)
Control	–
1	28.6 ± 0.1^{d}
2	29.8 ± 0.2^{d}
4	32.5 ± 1.2^{c}
8	35.6 ± 1.4^{b}
12	37.2 ± 0.6^{a}
16	39.8 ± 1.3^{a}

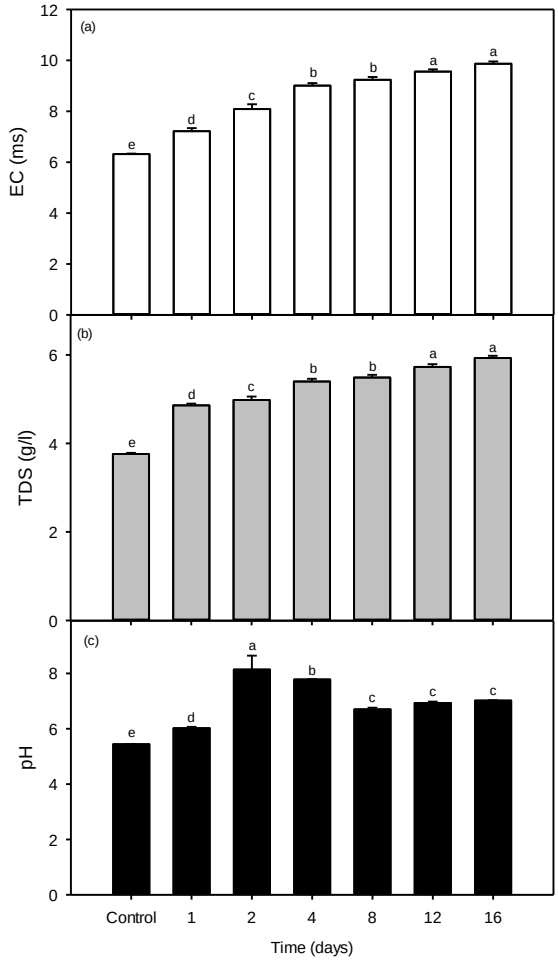

Figure 3. Three parameters measured to assess anaerobic digestion culture conditions, across six incubation time periods (1, 2, 4, 8, 12, and 16 days). (**a**) electrical conductivity (EC), (**b**) total dissolved solids (TDS), and (**c**) pH. Different letters indicate ED, TDS, and pH values that are significantly different ($p < 0.05$).

4. Discussion

Of the six weed species we tested under the AD conditions imposed in this study, seeds of *S. purpurea* and *A. viridis* had the highest levels of thermal resistance. At 8 days of incubation, seeds of *S. purpurea* and *A. viridis* germinated relatively rapidly: germination took five days and eight days, respectively. In addition, the percentage of seeds that germinated for both species remained relatively high over the first four incubation time periods, especially for *A. viridis*, with 35.2% of seeds germinating at 8 days of incubation. Even though no seeds of *S. purpurea* and *A. viridis* germinated at 12 and 16 days of incubation, their patterns of percent of viable and non-viable seeds were similar; some seeds were

still viable at 16 days of incubation, with 32.5% of *S. purpurea* seeds remaining viable at 16 days. Our results are generally consistent with those previously published for both species. Mature seeds of *A. viridis* are initially dormant, with germination reaching its highest levels after two months at a temperature of 30 °C [48]. In addition, germination of *A. viridis* is completely inhibited by high osmotic potentials [48], while seed germination of *S. purpurea* is inhibited by high salinity [49]. Whether viable seeds of both species are still capable of germinating is unknown and assessing this will be the focus of future research. However, the percentage of viable seeds for both species declined over the 16 days of incubation. Therefore, higher seed mortality will likely occur if AD is conducted for more than 16 days, as in these experiments.

Both *T. aestivum* and *S. bicolor* are members of Poacea, are cultivated crop plants, and under some circumstances, are considered undesirable crop species in the region near Qena, Egypt. Both species exhibited very similar germination and viability results over the 16 days that AD was conducted. Seeds of both species germinated in one day, with their time to first germination increasing to nine days at 8 days of incubation (no seeds germinated at 12 and 16 days of incubation). Both had 100% and 97.8% germination, respectively, and the percentage of seeds that germinated for both species decreased steadily over the first four incubation time periods. The percentage of viable seeds for both species decreased over the first five incubation time intervals, and no viable seeds were detected on day 16. Most cultivars of *T. aestivum* have low levels of seed dormancy, in which seeds experience an after-ripening period, after which they lose dormancy [50]. Mature dormant seeds of *S. bicolor* can be stimulated to germinate simply by incubating them at high temperatures (e.g., 30 °C) [51]. Grasses tend to exhibit low survival rates during AD, although variation among grass species does occur [1,52]. Our results for *T. aestivum* and *S. bicolor* are generally consistent with these previous reports and indicate low dormancy levels and complete seed mortality by 16 days of AD.

Overall, *P. equisetiforme* and *L. arabicus* exhibited the poorest seed germination results over the 16 days that AD was conducted. Control seeds of both species germinated in two days. The time to first germination for *P. equisetiforme* was 17 days at 2 days of incubation and no other seeds of this species germinated during the last four time periods of the study. The time to first germination for *L. arabicus* was 20 days at 4 days of incubation and no other seeds of this species germinated during the study. The control seeds of *P. equisetiforme* had 86.7% germination, while only 10.0% of *L. arabicus* control seeds germinated. The percentage of viable and non-viable seeds were similar for both species, with a low percentage of seeds still viable at 16 days of incubation. To the best of our knowledge, no studies have been published assessing seed dormancy and germination of *P. equisetiforme* and *L. arabicus*, especially after AD. However, seeds of other *Polygonum* species that have been exposed to AD incubation at >40 °C for 20 days or more typically have low survival rates or are inactivated [1]. Because members of Fabaceae possess seed coats with a water-impermeable layer, they have high dormancy and low rates of germination, even under favorable conditions [53]. This may explain the seed germination and viability patterns we observed for *L. arabicus*.

Survival of seeds during AD is highest for seeds with a water-impermeable layer in the seed coat (i.e., high seed coat hardness or physical dormancy) or other protective structures [1,6,52]. This water-impermeable layer in the seed coat prevents seeds from imbibing water, at least early in the AD process, and maintains seeds at a low moisture content and at low metabolic activity, thus protecting seeds from thermal inactivation (such seeds are thermoresistant). For seeds with low seed coat hardness, just the opposite characteristics hold true, and such seeds have high mortality rates during AD. Hard seed coats are associated with the species in the Convolvulaceaea, Fabaceae, Geraniaceae, Malvaceae, and Solanaceae, although species in these plant families vary for this characteristic and thus their thermal resistance properties [6]. Therefore, it is difficult to know or predict the speed and amount of inactivation that seeds of various plant species will experience without conducting AD trials, as reported here.

The survival, germination, and viability of seeds during AD are determined by a wide range of factors, including the length of time (i.e., the duration) AD is allowed to occur, the operational temperature of the AD process, and the pH of the AD conditions [1,4–7,9,10,52]. During the AD process, the germination and viability of seeds decrease exponentially over time; thus, as AD is conducted for longer periods of time, seed mortality increases [1,6,52]. Moreover, the exponential inactivation of seeds is usually preceded by a lag phase, and temperature is the most important factor influencing this lag phase [1]. With higher incubation temperatures during AD, the lag phase is shorter, and the inactivation of seeds occurs at a faster rate. With respect to incubation temperature, two types of AD can be conducted: mesophilic AD (35–37 °C) and thermophilic AD (55–60 °C) [1,54–56]. During mesophilic AD, the incubation process must be conducted for a longer period to achieve similar seed mortality levels as thermophilic AD conducted for shorter periods of time. While thermophilic AD has certain advantages, such as causing faster and higher rates of seed mortality (and higher biogas production) [1], mesophilic AD is preferred under some circumstances because it is easier to maintain and is more stable than thermophilic AD [54–56]. Seed survival during AD is highest when neutral pH values (6.8 to 8.0) are maintained and is lowest at extreme pH values, especially in extremely acidic conditions, which occurs with substrate overloading [1,7].

In this study, four parameters were measured at each incubation time interval: temperature, electrical conductivity (EC), total dissolved solids (TDS), and pH. For the reasons mentioned above, AD was conducted at a temperature range consistent with mesophilic AD (28.6–39.8 °C). As often occurs, temperatures increase with the increasing duration of AD incubation. These increases in temperature likely contributed to the decreases in seed germination and viability observed in this study. EC is a measure of a fluid's (water's) ability to conduct electricity, and is related to the total concentration, mobility, valence, and relative proportion of ions in solution. TDS is determined by e concentration of any mineral, salts, metals, cations, or anions dissolved in water. Both EC and TDS values increased steadily and significantly over the time that AD was conducted; the highest values of both parameters occurred at 12 and 16 days of incubation. The seed germination and viability patterns we observed for these six plants may, in part, be due to increases in the value of temperature, EC, and TDS, over the course of these experiments. This may be especially true for the seed germination of *A. viridis*, which is completely inhibited by high osmotic potentials, and seeds germination of *S. purpurea*, which is inhibited by high salinity [48,49]. Though pH values varied over the time that AD was conducted, their values never deviated from the optimum temperature for seed germination. Thus, pH values likely did not affect the seed germination and viability parameters reported here.

5. Conclusions

The digestate produced from AD can be used as a source of nutrient-rich, slow-release bio-fertilizer. However, if plant seeds survive the AD process and are released into the environment, they may proliferate and cause or worsen weed infestations. The six common plant species we analyzed showed highly significant statistical differences for almost all the seed germination and viability parameters we measured across the six time periods assessed in this study. For all six species, no seeds germinated at 12 and 16 days of AD. However, for four of the species we tested, *S. purpurea*, *P. equisetiforme*, *A. viridis*, and *L. arabicus*, some seeds remained viable after 16 days of AD. Whether viable seeds of these four species are capable of germinating was not determined in this study but assessing this will be the focus of future research. Future research should also assess other factors contributing to plant seed contamination in bio-fertilizer derived from anaerobic digestate: the abundance of plant seeds in the substrate used in the digestion process and the interaction of AD conditions (e.g., duration and operational temperature) and seed characteristics (seed coat hardness, and the hydration level, metabolic rate, and dormancy mechanisms). Overall, these results suggest that the application of anaerobic digestate in the reclaimed agricultural lands near Qena, Egypt, is not likely to exacerbate weed infestations in these fields.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020396/s1, Table S1: One-way ANOVA results for all four seed germination and viability measures that are presented in Tables 1 and 2 and Figure 2, for the controls and each of the six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days); Table S2: Percent of seeds that germinated for the six plant species, across six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). This table contains the numerical data presented in Figure 2; Table S3: One-way ANOVA results for the three culture condition parameters, across six anaerobic digestion incubation time periods (1, 2, 4, 8, 12, and 16 days). This table shows the statistical analysis of the data presented in Figure 3.

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