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Seed germination test for toxicity evaluation of compost: Its roles, problems and prospects

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ABSTRACT

Compost is commonly used for the growth of plants and the remediation of environmental pollution. It is important to evaluate the quality of compost and seed germination test is a powerful tool to examine the toxicity of compost, which is the most important aspect of the quality. Now the test is widely adopted, but the main problem is that the test results vary with different methods and seed species, which limits the development and application of it. The standardization of methods and the modelization of seeds can contribute to solving the problem. Additionally, according to the probabilistic theory of seed germination, the error caused by the analysis and judgment methods of the test results can be reduced. Here, we reviewed the roles, problems and prospects of the seed germination test in the studies of compost.

Contents

1. The roles of the seed germination test for the toxicity evaluation of compost

Composting, an effective and economical biotechnology, is widely used in the sanitization and recycling of biowastes including animal manures (Ge et al., 2016; Liang et al., 2017a) and carcasses (Gwyther et al., 2011), crop straws (Zhang et al., 2016), agro-industrial residues (Aviani et al., 2010), municipal organic solid wastes (Kelessidis and Stasinakis (2012); Anand and Apul, 2014), etc. Compost is often used as the growth media (Boldrin et al., 2010), the organic fertilizer (Feng et al., 2016), the soil amendment (D Hose et al., 2014; Alvarenga et al., 2015) and the suppressive substance of soil-borne plant diseases (Mehta et al., 2014; Yu et al., 2015) for agricultural production and landscaping. In recent years composting has been studied for the bioremediation of soils contaminated with heavy metal(loid)s (Park et al., 2011; Wu et al., 2016; Liang et al., 2017 b) and organic

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contaminants (Chen et al., 2015; Huang et al., 2016; Kastner and Miltner, 2016).

Compost quality, such as stability and maturity, should be checked before the application of compost on land. The unstable and/or immature compost can have adverse effects on seed germination, plant growth and soil environment due to the decreased supply of oxygen and/or available nitrogen or the presence of phytotoxic compounds (Bernal et al., 2009). Stability is the resistance of the organic matter in compost against further microbial decomposition as long as there is no inhibition on the microbes by other factors not relevant to the organic matter, while maturity is an agronomic parameter that is clearly associated to the effect of compost on plant growth (Komilis, 2015). The indices of respiration and humification of compost are used to evaluate the stability and maturity of compost (Komilis and Kanellos, 2012; Hill et al. (2013); Nikaeen et al., 2015), respectively. The substances, including low molecular weight organic acids (e.g. phenolic acids (Marambe et al., 1992)), ammonium nitrogen (NH_4^+-N) (Ramírez et al., 2008), salinity (Hase and Kawamura, 2012), heavy metals (Fuentes et al., 2004), xenobiotics (e.g. antibiotics (Liu et al., 2009) and agrochemicals (Cayuela et al., 2008; Tang et al., 2008)), can cause damage to plants when they are in high levels. In general, many of these substances need to be gauged via time-consuming and expensive detection processes to determine whether their levels are beyond or within acceptable ranges. However, there exists the possibility for the unexpected factors that are not taken for analysis. Furthermore, there is a lack of analytical procedure to evaluate the joint effect of the toxic substances in compost. Consequently, as a bioassay, seed germination test has attracted a lot of attention to overcome these concerns. The seed germination index (*GI*) was firstly proposed by Zucconi et al. (1981) who used cress seeds in the germination test for evaluating the toxicity of compost. *GI* is calculated by the radicle length and germination percentage of the seeds in the sample (compost extract) compared to that in the control (e.g. deionized water). *GI* is correlated with some other biological and chemical indices for evaluating compost quality. The study of El Fels et al. (2016) showed that *GI* was positively correlated with the biological index of the *Artemia salina* cytotoxicity test for evaluating the toxicity of compost. In addition, *GI* is positively correlated with humification parameters (Gavilanes-Terán et al., 2016) while negatively correlated with the content of NH_4^+ (Tiquia et al., 1996; Guo et al., 2012). Therefore, the seed germination test has been broadly accepted for evaluating compost quality. In Italy, *GI* is listed in the quality assessment regulation of compost for commercialization (Cesaro et al., 2015). However, there are large variations in the aspects of method and seed species of the test among previous studies (see Section 2). The problems and prospects of studies in the seed germination test will be comprehensively reviewed in the following parts.

2. Could there be a widely accepted procedure of the seed germination test?

The seed germination test procedure consists of three major steps. Firstly, prepare an aqueous extract of compost; secondly, incubate seeds with the extract; thirdly, measure and calculate the indicators related to the test results by Eqs. (1–6), including the seed germination (*SG*), the relative seed germination (*RSG*), the relative radicle growth (*RRG*) and the seed germination index (*GI*). More details of the typical test procedures in studies are summarized in Table 1.

$$SG = \frac{\text{Number of germinated seeds}}{\text{Number of total seeds}} \times 100\% \quad (1)$$

$$RSG = \frac{\text{Number of germinated seeds(sample)}}{\text{Number of germinated seeds(control)}} \times 100\% \quad (2)$$

$$RRG = \frac{\text{Total radicle length of germinated seeds(sample)}}{\text{Total radicle length of germinated seeds(control)}} \times 100\% \quad (3)$$

$$GI = RSG \times RRG \times 100\% \quad (4)$$

$$GI = \frac{GI_{50\%} + GI_{75\%}}{2} \times 100\% \quad (5)$$

$$GI = \frac{GI_{25\%} + GI_{50\%} + GI_{75\%}}{3} \times 100\% \quad (6)$$

where $GI_{25\%}$, $GI_{50\%}$ and $GI_{75\%}$ were *GI* values of the samples that were 25%, 50% and 75% of the raw aqueous extract of compost diluted with deionized or distilled water (v/v), respectively.

At present, the major problem in the studies of the seed germination test is that there is no universally adopted procedure, which is reflected at the incompleteness of the test procedure and the differences of the corresponding methods (Table 1). In the first step, it is essential to determine the extraction ratio because the toxicity of compost extract is related to its concentration (Emino and Warman, 2004; Said-pullicino et al., 2007; Young et al., 2016). Although the extraction ratio of 1:10 (w/v) is one of the most used ratios in studies (Table 1), the test samples are fresh samples and have different moisture content. Owing to the large change in the moisture content of the raw material during composting, the moisture content of the samples collected from different composting stages is different (Chikae et al., 2006). In order to accurately evaluate the change of the toxicity of samples during composting and increase the comparability of the toxicity of samples with different moisture content, it is necessary to eliminate the interference of moisture content on test results. So far, there are two methods to address this problem. One method (Chikae et al., 2006; Khan et al., 2014) is to prepare the extract according to a certain extraction ratio of water and dry weight of fresh sample, but how to select a suitable value of the extraction ratio remains unsettled. Another method (Pampuro et al., 2010) is to adjust the moisture content of fresh sample to 85% to prepare the extract or its dilutions for the seed germination test (Said-pullicino et al., 2007; Cesaro et al., 2015). Centrifugation and membrane filtration can quickly and effectively remove the particulates in the extract. They are essential steps to reduce the interference of particulates on test results. In the second step, the major differences are reflected at the species of seed and the definition of seed germination (Table 1). There is no recognized seed species that can be used to evaluate the toxicity of compost and the seed is often obtained locally. The definition of seed germination can be divided into three groups by radicle length: only visible, at least 2 mm and at least 5 mm. It is not difficult to observe, even the incubation time is prolonged, the initial radicles of the germinated seeds inhibited in test are no longer to elongate, so a certain length that the radicle reached is used as the operational definition of germination. In fact, the process of germination is completed by visible radicle protrusion through the testa (seed coat), and radicle elongation belongs to post-germination (Weitbrecht et al., 2011). Moreover, if the incubation time is too long, the secondary root will begin to grow and the cotyledon will have strong phototaxis, and the length of radicle cannot exactly reflect the toxicity. The incubation time depends on specific environmental conditions and seed species. After the incubation time is over, seeds can be frozen at -10 °C for 24 h or added with ethanol equivalent to the sample (aqueous extract) to end their growth (Macias et al., 2000; Gómez-Brandón et al., 2008). These methods are effective in the handling of seeds,

Table 1
The essential steps of the seed germination test for evaluating the toxicity of compost.

Primary feedstocks of compost	Aqueous extract				Seed germination					References
	Sample to water ratio (w/v)	Shake	Centrifugation	Filtration	Species of seed[d]	Number of seeds per Petri dish	Volume of extract (mL) per Petri dish	Incubation time (d)	Operational definition of germination	
Municipal solid waste, yard trimmings, foliage residues	Dilutions (25%, 50% and 75% in deionized water) of the aqueous extract of the fresh sample with 85% moisture content (wet weight)	Without shaking, standing and contacting 2 h	10 000 rpm for 10 min	0.45 μm membrane filter	*Lepidium sativum* L.	10	1	2	-[e]	Said-pullicino et al. (2007)
Municipal solid waste	Dilution (30% in deionized water) of the aqueous extract of the fresh sample with 85% moisture content (wet weight)	Without shaking, standing and contacting 2 h	6000 rpm for 15 min	Sterilizing membrane	*Lepidium. sativum*, *Sorghum. saccharatum*, *Cucumis. sativus*	10	3	3	≥ 0.5 mm	Cesaro et al. (2015)
Green waste	1:5[a]	With shaking for 24 h	–	Qualitative filter paper	Pakchoi (*Brassica rapa* L., Chinensisgroup)	20	1	2	–	Zhang et al. (2013)
Vegetable residues	1:10[a]	200 rpm for 40 min	6000 rpm for 15 min	0.45 μm millipore filter paper	Radish	10	10	2	–	Huang et al. (2016)
Food waste	1:10[b]	With shaking for 30 min	–	Filtrated	Komatsuna (*Campestris brassica*)	30	10	2	–	Chikae et al. (2006)
Food waste	Fresh sample	–	–	–	Cress ('Peppergrass')	20	–	5	A visible radicle	Aslam et al. (2008)
Kitchen waste	Sample submerged by water	–	–	–	Cress (*Lepidium sativum*)	10	10	2	–	Zeng et al. (2007)
Kitchen waste	1:10[a]	150 rpm for 30 min	4000 rpm for 20 min	0.45 μm membrane filter	Pakchoi	20	10	3	–	Yang et al. (2013)
Pig manure	1:10[a]	10 min	–	Qualitative filter paper	Chinese cabbage, Chinese kale, Chinese spinach, cucumber, onion, tomato	10–30	10	5	≥ 5 mm (radicle length)	Tiquia et al. (1996)
Pig manure	85% moisture content (wet weight)	2 h	6000 rpm for 15 min	Filtrated	*Lepidium sativum* L., *Raphanus sativus* L., *Sinapis alba* L.	10	1	1–3	A visible radicle	Pampuro et al. (2010)
Pig manure	1:10[a]	150 rpm for 1 h	4000 rpm for 20 min	0.45 μm membrane filter	Cucumber	10	8	2	–	Guo et al. (2012)
Pig manure	1:10[a]	150 rpm for 1 h	4000 rpm for 20 min	0.45 μm membrane filter	Radish	20	10	2	–	Zang et al. (2016)
Dairy manure, beef manure, pig manure	1:2[c]	1 h	3000 rpm for 20 min	Filtrated	Radish (*Raphanus sativs* L.)	30	10	3	≥ 5 mm (radicle length)	Ko et al. (2008)
Chicken litter	1:10[b]	200 rpm for 3 h	3000 × g for 20 min	0.45 μm syringe filter	Cress (*Lepidium sativa*)	8	1	3	–	Khan et al. (2014)

[a] With fresh samples.
[b] With fresh samples by dry weight basis.
[c] With dry samples.
[d] The seed germination temperature was between 20 °C and 25 °C, which was suitable for seed germination, so the temperature was not listed.
[e] The step was absent.

especially in measuring the radicle length of germinated seeds accurately. The third step is mainly about the calculation of *GI* (see Eqs. (2–4) for more details). The *GI* value, not less than 80%, usually means that compost has no phytotoxicity (Tiquia et al., 1996). However, the toxicity of compost is also affected by the extraction ratio. One way to eliminate the effect of the extraction ratio is preparing high concentration of compost extract and using its dilution with water (v/v) for the test, so the actual toxicity of it could be reflected by *GI*. Said-pullicino et al. (2007) enhanced the moisture content of the fresh compost samples to 85% (wet weight) with deionized water and made aqueous extracts by contaction, centrifugation and filtration (see Table 1 for more details). The results showed that the values of *GI* increased from 12.8% (day 0) to 74.7% (day 250). However, the electrical conductivity values of the composting mixture samples were from 5.0 to 7.8 mS cm^{-1}, which implied that the concentrations of soluble salts were in the level considered being inhibitory for seed germination (Hoekstra et al., 2002). In addition, as seen from Eqs. (4–6), the accuracy of *GI* to evaluate the toxicity of compost could be improved by increasing the weight of *GI* values of different dilutions. Mitelut and Popa (2011) prepared compost extract with the fresh sample and distilled water at the ratio of 1:2 (w/v), then it was diluted with distilled water to yield 0%, 25%, 50%, 75% and 100% of the initial extract (v/v). The global germination index (Eq. (5)), i.e. the average of *GI* values of the 50% and 75% of the extracts, was adopted in their study. The results showed that the index values of the samples were below 80% and the germination percentages of the extracts (beyond 25% of the initial extract) were almost zero, which proved to be a high phytotoxicity of the samples. Furthermore, the study revealed that the samples significantly inhibited seed germination (radicle emergence), and let alone radicle elongation. Similarly, according to the test procedure listed in Table 1 and Eq. (6), the results of Qian et al. (2014) showed that the *GI* values varied from 68% (day 30) to 129% (day 90) in swine manure composting and from 88% (day 30) to 119% (day 90) in dairy manure composting. In addition, the *GI* values of commercial compost of swine manure and dairy manure reached 145% and 126%, respectively. Recently, Young et al. (2016) made the extract concentrations used in the test range from 0.5% to 100% (i.e. 0.5%, 1%, 5%, 10%, 20%, 40%, 60%, 80% and 100%, v/v) of the raw extract that was prepared by mixing a dry sample with deionized water (1:10, dw/v). They proposed that the two concentrations of compost extract (*RRG*=80% and *GI*=80%) were both over 100%, which indicated non-inhibitory effect of compost. This is a new idea to study the phytotoxicity of compost from the perspective of dose-effect relationships, and it has important guiding significance for the comparison between samples with different toxicity degrees.

3. Is it necessary to improve analysis and judgment methods of the results of the seed germination test?

Seed germination is a random event. With the increase in the number of test seeds, the occurrence frequency of germinated seeds will gradually approach a stable value, i.e. the probability (*P*) that represents the viability of the whole selected seeds. Generally, the germination frequency or percentage of one thousand seeds is adopted as *P*. Assuming a commercial seed lot with the germination percentage of 90% is used to carry out the test with ten seeds per Petri dish. It is well known that the probability of the number of the germinated seeds are subject to binomial distribution (Boyle, 2003). Thus, solving for the binomial distribution of ten seeds per Petri dish (see Table 2 for more details), results indicate that nearly 99% of Petri dishes would contain seven to ten germinated seeds, and there is low probability (<0.0001) of the germinated seeds less than five in a Petri dish. According to the principle of the small probability event ($\alpha = 0.05$), the case that the number of germinated seeds is less than seven in ten seeds does not occur in one test ($P < 0.05$). If it does in the Petri dish of the sample, which means that the seed germination is inhibited; if it does in the Petri dish of the control, which means that the seed selected is undesirable for the test. Usually, plump and shiny seeds are sieved to ensure the germination under suitable conditions, but there may be some special ones, such as the deep dormant seeds (Derek Bewley, 1997), which still cannot germinate. Therefore, the principle of probability and statistics to judge the number of germinated seeds in test can reduce the error caused by the special seeds.

There are currently three viewpoints on analysis and judgment of the results of the seed germination test. First, *GI* is widely adopted because it combines *RSG* with *RRG*, both of which can reflect the toxicity of compost (Zucconi et al. (1981); Emino and Warman, 2004). Second, *RRG* is more sensitive indicator than *RSG* to the toxicity (Tiquia et al., 1996; Fuentes et al., 2004), so *RRG* is used alone. Third, the toxic level of the compost that inhibits seed germination is higher than that inhibits radicle elongation, thus *RSG* and *RRG* are used to evaluate the toxicity separately. In other words, if compost inhibits seed germination, it is not necessary to evaluate the effect of it on radicle elongation; if not, the effect on the radicle needs to be evaluated. Luo et al. (2016) used the indices of *SG* (Eq. (1)), *RRG* and *GI* to determine the profile of the toxicity of pig manure during composting. The results showed that the *RRG* values of two species of seeds (radish and cabbage) were significantly and positively correlated, and the *SG* values of all seeds were more than 70% (seven germinated seeds of ten seeds per Petri dish), which indicated that compost had no effect on germination of the seeds. The third viewpoint on the test is worth studying in compost derived from other biowastes.

Moreover, time courses of seed germination are usually several days under suitable conditions, which can be morphologically divided into three phases (Fig. 1) that consist of phase I (imbibition), phase II (radicle emergence) and phase III (radicle elongation). The uptake of water is the major process of seed germination during the phase I, which could be negatively affected by high salinity of compost. During the phase II, the low molecular weight organic acids of compost could be the primary inhibitor of radicle emergence after testa rupture. Radicle elongation could be inhibited by NH_4^+ during the phase III. This speculation partly supports a viewpoint that seed germination can be used to examine the compost with high toxicity and radicle growth can be used to examine the compost with low toxicity (Zucconi et al. (1981); Tiquia et al., 1996). Further studies are needed to validate the effectiveness and applicability of this conception in the seed germination test.

Table 2
Binomial probability distribution of the number of germinated seeds with ten seeds per Petri dish.[a]

Number of germinated seeds	Binomial formula	Probability (*P*)
10	$C_{10}^{10}p^{10}(1-p)^0$	0.3487
9	$C_{10}^{9}p^{9}(1-p)^1$	0.3874
8	$C_{10}^{8}p^{8}(1-p)^2$	0.1937
7	$C_{10}^{7}p^{7}(1-p)^3$	0.0574
6	$C_{10}^{6}p^{6}(1-p)^4$	0.0112
5	$C_{10}^{5}p^{5}(1-p)^5$	0.0015
4	$C_{10}^{4}p^{4}(1-p)^6$	0.0001
3	$C_{10}^{3}p^{3}(1-p)^7$	8.75×10^{-6}
2	$C_{10}^{2}p^{2}(1-p)^8$	3.64×10^{-7}
1	$C_{10}^{1}p^{1}(1-p)^9$	9.00×10^{-9}
0	$C_{10}^{0}p^{0}(1-p)^{10}$	1.00×10^{-10}

[a] Assuming an infinitely large population of seeds where 90% are capable of germinating ($p = 0.9$).

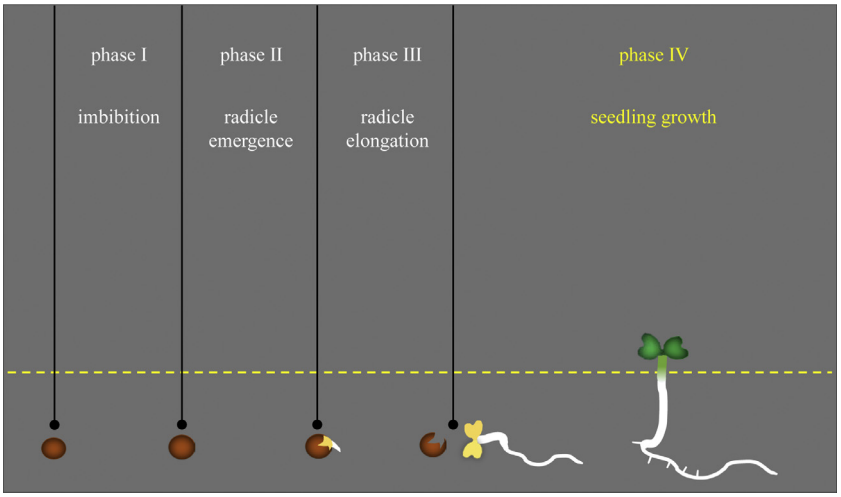

Fig. 1. Morphological key processes during the seed germination and seedling growth of Chinese cabbage, a non-endospermic eudicot. The yellow and white fonts represent the stage where light is and is not required, respectively. The dotted line indicates the top of the dish for placing seeds. The diagram is based on the theories of Derek Bewley (1997) and Weitbrecht et al. (2011) and the study of Luo et al. (2016).

4. Could there be a species of plant seed to be used as a model seed for the toxicity evaluation of compost?

The result of the seed germination test is a seed species-dependent parameter, and cress seed is the most adopted species in previous studies (Said-pullicino et al., 2007; Zeng et al., 2007; Aslam et al., 2008; Pampuro et al., 2010; Khan et al., 2014). However, the range of application of cress seed is proved to be limited, and there has been a few works to determine whether there exists a species of plant seed that is more sensitive than cress seed to the toxicity of compost. Emino and Warman (2004) compared the *GI* values of mature and immature municipal solid waste compost with three groups of the seeds that included the large size (green bean, sweet corn, hybrid cucumber and sunflower), the medium size (broccoli, Chinese cabbage, radish, tomato, amaranthus and shasta daisy) and the small size (cress, carrot, lettuce and petunia) seeds. They concluded that most of the species, including cress seed, were not sensitive enough to discriminate between the mature and the immature compost, while Chinese cabbage seed was the most sensitive one of these seeds to that. Most importantly, they firstly proposed a criterion that the model seed would germinate and grow well in mature compost, whereas the seed would germinate less and grow slowly in immature compost. Earlier studies of Chinese cabbage seed in pig manure composting (Tiquia et al., 1996) and three types of mature compost (Warman, 1999) indicated that Chinese cabbage seed was sensitive to the low level of the toxicity of compost, which confirmed the conclusion that Chinese cabbage seed met the criterion. Recently, Zhang and Sun (2016) used the germination test of Chinese cabbage seed to evaluate the toxicity of compost made from lignocellulosic waste. In addition, Chinese cabbage is a subspecies of *Brassica rapa* (Saeki et al., 2016), and Komatsuna, a variety of *Brassica rapa*, is related to Chinese cabbage. The germination test of Komatsuna seed had been widely used in compost (Hase and Kawamura, 2012). Chinese cabbage seed has its own advantages in terms of the response to toxic substances (sensitive), the germination cycle ($\leq$ 48 h) and the seed size (medium), so it could be used as a model organism to investigate the toxicity of compost.

5. Conclusions and prospects

Seed germination as the critical initial stage of plant growth has its own performances against seedling growth, such as the sensitivity to environmental pollution and the short-period course without photosynthesis. The seed germination test is an effective and economical bioassay to evaluate the potential toxicity of compost before it can be used. Further studies of the determination of the extraction ratio (e.g. sample to water, dw/v), the application of the probabilistic theory (e.g. binomial probability), the correlation between affecting factors (e.g. salinity, low molecular weight organic acids and NH_4^+) and different stages of seed germination process (imbibition, radicle emergence and radicle elongation), and the selection of model seeds (e.g. Chinese cabbage) in the seed germination test of the compost made from different kinds of feedstocks are still required. Moreover, future investigations are needed to evaluate and correlate the different procedures of the seed germination test. Overall, the standardization and improvement of the test procedure and the modelization of the test seeds are beneficial to enhancing the validity and reproducibility of the seed germination test for evaluating the toxicity of compost.

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