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Reliability of Bioassay Tests to Indicate Herbicide Residues in Compost Of Varying Salinity and Herbicide Levels

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A variety of bioassay methods have been developed recently employing plant visual injury symptoms to indicate presence of auxinic herbicide residues that may occur in certain composts (yard trimming composts, for example). These various methods differ appreciably with regard to selected plants and the means of preparing test media. Varying salinity, pH and maturity of composts are known to influence seedling growth, with the possibility of incorrect attribution of harmful effects. Therefore, the need exists to adequately exclude confounding effects for reliable and reproducible bioassays. This study employed peas and red clover of moderate and high sensitivity to auxinic herbicides, respectively, and compared two differing bioassay protocols, one which prescribes 67% of compost in the test medium (Procedure 1), and the other which adjusts compost percentage in the medium based on test salinity (EC) of the initial material (Procedure 2). Composts with three levels of EC (4.2, 12.4, and 20.6 dS m^{-1}) and two levels of clopyralid (10 and 50 ppb) were evaluated by rank-ordering of visual injury, and by fresh yield biomass, at 17 days after planting. Fresh plant weights for peas and clover were not correlated with clopyralid content of compost media at any EC level. Visual injury symptomology was greater in clover than peas for both procedures at all combinations of compost EC and clopyralid content. For low and medium EC composts both procedures identified 10 and 50 ppb clopyralid in the compost, but only Procedure 2 was able to do so at the high EC level. Both protocols failed to distinguish low clopyralid at the highest EC levels when employing peas. Only Procedure 2 with clover was capable of distinguishing both levels of clopyralid at all levels of compost EC. The test medium salinity in Procedure 1 using the high-EC compost was high enough to account for this difference.

Introduction

A recent study evaluated the effects of compost salinity on the performance of plants often used in herbicide bioassays and demonstrated that salinity and pH of compost test media significantly influenced plant growth, leading in some cases to stunting, yield-reduction and total-loss of seedlings (Brinton, *et al.* 2005). The principal cause of harmful effects was shown to be salinity with secondary effects from pH. Recently, residues of clopyralid (3,6-dichloropicolinic acid) herbicide in composts made from grass clippings were detected in 1999 when reports in Washington indicated herbicide damage to plants from use of certain composts (Fauci *et al.* 2002; Michel and Doohan, 2003, Nielsen 2003). Turf clipping studies showed significant but not total dissipation of residues following application and prior to recycling (Miltner *et al.* 2003). Pesticide residues are reported to degrade variably in composts (Bugbee and Saraceno 1994; Michel and Doohan, 2003; Vandervoort *et al.* 1997). Recent laboratory trials have shown that clopyralid dissipated during composting with a half-life of 9 -102 days (Brinton *et al.* 2004; Ronald H. Turco, personal communication, Purdue University, July 2003), similar to soil dissipation results (Ahmad *et al.* 2003). However, composts may be considered market ready and distributed between 30 and 300 days (Spalvins 2003; Blewett *et al.* 2005). Therefore, the need exists for reliable bioassays to evaluate the potential for plant damage from herbicide residues and to accurately distinguish these potential effects from other factors in compost that may exert similar negative effects.

Plant bioassays have been used widely in weed research to determine the concentration of herbicides in soil or growing media (Santelmann *et al*. 1971; Agdex 2001). Although less specific than chemical tests, they can be useful in detecting phytotoxic metabolites not detected by chemical methods, and are relatively low cost. A variety of bioassay methods have been developed very recently to detect significant auxinic herbicide residues in compost (Bary and Cogger 2002; Fauci

2003; Fauci *et al.* 2002; KCSW 2002; SPU 2002; WA-DOE; 2003; WERL 2003). A common feature is that the methods employ various ratios of compost blended with other growing media. It is generally accepted that some dilution of the compost is required to procure a satisfactory medium to allow good seed germination. However, it is also apparent from the high ratios of compost employed by most of these procedures that the goal is to maximize compost use in order to achieve the greatest likelihood of detecting herbicide residues which may be present in the ug kg^{-1} range (Fauci 2003). In contrast to composts, direct soil bioassay methods normally employ straight (100%) soil (Rashid *et al.* 2001; Agdex 2001). Indirect bioassay methods for environmental samples may employ soil-sand mixtures or water extracts of varying ratios, particularly if the goal is to distinguish the modus of inhibition and the class of herbicide residue (ASTM 1999; Böger and Sandmann 1993). Published criteria for plant toxicant studies require documentation of potential interference to the procedure (ASTM 1999), and work with composts has indicated that such interferences do exist (Morel and Guillemain 2004; Brinton and Evans 2002). Normally, to quantify background phytotoxicity not attributable to an auxinic source, a nontarget plant would be employed. The Cruciferae species, *Lepidium sativum* (Garden cress) is a plant with resistance to several auxinic herbicides, and is listed under OECD (Organization for Economic Cooperation and Development) and ASTM (American Society of Testing Materials) for routine phytotoxicity testing (OECD 2003; ASTM 1999). Moreover, cress has been extensively used to evaluate composts generally for plant performance in Germany and Switzerland (Fuchs *et al.* 2001; Kehres and Pohle 1998). However, we are aware of no efforts to distinguish herbicides from each other in compost bioassays nor are we aware of any attempts to rule out background phytotoxicity with a nontarget plant responsive to compost performance.

Use of 100% compost in growing media for bioassays is problematic, owing partly to variable and potentially high salinity as measured by electrical conductivity (EC). We previously surveyed 39 Washington State green composts and found a mean EC of 6.2 dS m^{-1} with a range from 0.4 to 24.2 dS m^{-1} with 64% of samples with EC greater than 3.5 dS m^{-1}, by saturated paste method. Therefore, most of the measured ECs were high enough to induce performance problems if used without significant dilution prior to planting. A King County, Washington report surveyed clopyralid levels in compost and found wide variation in apparent herbicide levels when quantitative lab analytical (GC/MS) was compared to results from plant bioassays. The report also described unclear threshold effects for plant damage that varied by matrix (KCSW 2002). An Oregon Department of Ecology survey showed widely varying thresholds of sensitivity to herbicide residues for one bioassay procedure when conducted at different time points for different compost samples (ODEQ 2003). In a previous paper, we showed that bioassays performed on composts that are not first corrected for EC of the media resulted in poor growth and stunting and led to the hypothesis that varying EC levels could affect bioassay performance in detecting phytotoxicity from herbicide residues (Brinton *et al.* 2005). In this study we evaluated the reliability of detecting plant damage from herbicide residues with and without adjusting for background EC, using composts of varying, known clopyralid content.

Material and Methods

Three compost composites were prepared by blending a selection of 12 west-coast (USA) municipal green waste composts having a range of EC between 1.6 to 17.1 dS m^{-1} determined by saturated paste method (USCC-REF, 2002). These composts were previously determined to be free of clopyralid by quantitative analysis and free of auxinic injury symptoms by plant bioassay. The blending resulted in three composites of high-, moderate-, and low-EC (20.6, 12.4, and 4.2 dS m^{-1}) respectively (Table 1). Acceptable EC levels in growing media for seedlings based on saturated paste methods are commonly considered to be in the range from 1.99 to 3.5 dS m^{-1}, in accordance to NCSU, MSU and Cornell guidelines (Bailey *et al.* 2004). Recently, 1:5 methods have been proposed for compost EC, and the corresponding 1:5 EC values were also determined (Table 1). We chose this range of salinity to bracket the range we previously observed in examining composts. Each of the three composite composts were subsequently spiked at two rates with compost that contained clopyralid at 400 ug kg^{-1} clopyralid (total solids-basis) to result in target levels of 10 and 50 ug kg^{-1} clopyralid in the compost media, for a total of six composite composts (Table 2). In order to obtain the compost with this level of clopyralid, compost was not spiked with 3,6-dichloropicolinic acid, but instead grass-clippings treated with specified amounts of her-

TABLE 1.
Salinity properties of compost blends used in the bioassay trials.

Compost Type	EC – sat. Paste	EC – 1:5 Method	pH Paste
Low EC	4.2	2.15	8.2
Moderate EC	12.4	3.75	8.2
High EC	20.6	7.42	7.8

TABLE 2.
Composition, predicted and measured properties of bioassay test media. A – compost EC type is described in Table 1; B – two clopyralid levels were targeted by spiking with compost containing 400 ug/kg clopyralid.

Procedure	Test Medium Designations Targeted Compost Properties EC [A]	Clopyralid ug/kg[B]	Test Medium Compositions, Test Compost By EC Type C Low	Mod.	High	Volume Percent Other Diluents Limed Peat	Potting Mix	Measured Properties In Test Media EC	pH	Clopyralid ug/kg
1	Low	10	67	0	0	0	33	2.2	7.37	12.5
2	Low	10	50	0	0	50	0	1.6	7.12	12.6
1	Low	50	67	0	0	0	33	3.1	7.60	49.3
2	Low	50	50	0	0	50	0	2.2	7.07	53.6
1	Moderate	10	0	67	0	0	33	5.5	7.72	7.1
2	Moderate	10	0	22	0	78	0	1.9	6.62	< 2.2
1	Moderate	50	0	67	0	0	33	7.1	7.75	32.7
2	Moderate	50	0	22	0	78	0	2.2	6.70	38.1
1	High	10	0	0	67	0	33	12.5	7.23	7.7
2	High	10	0	0	14	86	0	3.4	6.36	5.1
1	High	50	0	0	67	0	33	11	7.35	29.2
2	High	50	0	0	14	86	0	2.6	6.45	38.3

bicide were obtained (Miltner *et al.*, 2003), partially composted, and the resulting level of clopyralid in the compost validated by quantitative analysis.

The six compost media were subsequently subjected to two standard herbicide bioassay protocols, one developed by the Washington Department of Ecology (Procedure 1, WA-DOE 2003), and the other developed by Woods End Research Laboratory (Procedure 2, WERL 2003). In Procedure 1, test compost is blended in a prescribed 2:1 ratio with a peat-perlite potting medium, irrespective of the compost EC. In Procedure 2, compost is diluted with limed peat using a standard method to achieve a constant initial EC of 2.0 dS m^{-1} (USCC-REF 2002). Table 2 summarizes the volume composition of the various media and the targeted and measured EC and clopyralid levels.

Red clover (*Trifolium pratense*) and peas (*Pisum sativum*), both legumes sensitive to auxinic herbicides, were used as the test species. Red clover is a taxum listed for terrestrial plant toxicity protocols (ASTM 1999). The two species were planted in triplicate 150 cc pots in each of the 12 test media and a control medium consisting of Fafard® Germination Mix. *Pisum sativum*, var. Maxim, was planted 4 seeds per pot and *Trifolium pratense*, var. Mammoth, was planted 7 seeds per pot.

Two forms of plant response evaluation were employed including visual injury ranking and total biomass measurement at 17 days after planting (DAP). The visual appearance of the plants was compared with those in the control media, and ranked according to a pre-established plant injury scale assigning ordinal numbers for each of 5 increasing levels of visual injury (Table 3). This form of ranking is similar to previously described injury scales for auxinic symptomology; however, since the seedlings were relatively small, we did not divide injury symptomology between stems and leaves (Scuimbato et al, 2004). Severity levels of injury assessed by differing persons for randomized plants were averaged, from which rank-order correlation was performed later. Using this visual injury rank-order procedure, it was also possible to calculate the Spearman correlation coefficient, r_s, for differing individuals performing the visual evaluation process. Using this procedure, we determined that rankings of plant injury between trained individuals using this 5-level system are routinely highly correlated (r_s, >0.96). To tabulate plant effects and biomass, the quantity of emerged plant seedlings per cell were recorded to establish germination, and subsequently plants were harvested by cutting at the growing medium surface and immediately weighed, to 1 mg precision, to preclude any drying.

To obtain quantitative clopyralid information, chilled compost samples were shipped overnight and analyzed by Carbon Dynamics Institute, Springfield, Illinois. Samples were prepared in the laboratory by shaking in a caustic methanol solution, neutralized and purified via solid-phase extraction. The subsequent eluate was dried and derivitized with a 1-propanol:sulfuric acid solution, dried again and partitioned into hexane. The hexane extract was analyzed by GC/NCI-MS with a limit of quantitation (LOQ) of 1.0 ppb.

ANOVA (analysis of variance), regression and LSD data analysis were performed using the STAT Data Analysis Package (Perlman 1980), and Spearman rank-order coefficients calculated manually (Cohen and Holliday 1982).

TABLE 3.
Herbicide visual plant injury scale for 17-day seedlings grown in peat or peat-compost media.

Injury Rank	Observed Symptoms of Red Clover Seedlings at 17 DAP	Observed Symptoms of Field Pea Seedlings at 17 DAP
None (0)	Cotyledons fully emerged and either flat or very slightly curved downward. First true leaf (single) fully emerged, 8-12 mm diameter, round, and very flat, with 2-4 cm erect petiole. First compound leaf open and flat, or beginning to open.	Three to five leaves open. Open leaflets flat. Youngest leaflets folded in half, and flat. Stems erect and straight.
Slight (1)	Cotyledons and leaves normal size but slightly down-curled. Continued growth normal in size.	Youngest leaflets slightly curled upward at margin. Stems bent at some upper leaf nodes. Normal size, but continued growth declines
Moderate (2)	Cotyledons normal size but curled downward into half circle. Most first leaves strongly curled. Petioles normal size but curved and leaning. Little compound leaf development. Poor continued growth.	Most younger leaflets curled or folded upward. Lower leaflets mostly flat. Stems bent at most upper leaf nodes. Slightly stunted. Little further growth.
Severe (3)	Cotyledons stunted, curled into half or full circle. Leaves few, stunted, tightly curled, not erect. No further growth.	All leaflets very stunted and curled. Stems normal length, but bent at most nodes, and curved between nodes. No further growth.
Extreme (4)	Germination not reduced, but very stunted curled cotyledons. Most prostrate. No leaves. No further growth.	Leaves and stems very stunted and curled.

Results and Discussion

Test Media

The tested EC and clopyralid levels of the 12 test media agreed closely with the target levels while the pH values were moderately high in most composts (Table 2). After blending in a 2:1 volume ratio with Fafard® Germination Mix (source: Griffith Greenhouse Supply, Gray Maine) for the Procedure 1 method, and a variable amount of peat (limed with ground dolomite to pH 6.0) for the Procedure 2 method, these pH values fell, but remained higher in Procedure 1 (average pH 7.5) than in Procedure 2 (average pH 6.7) blends. The pH of growing media for chemical effects studies is generally targeted for 6.2 – 6.7 (ASTM 1999).

Germination

Germination of field pea with all composts in both procedures did not differ significantly from noncompost controls (Figure 1). However, using ANOVA a statistically significant interaction was observed for EC × Procedure × Clopyralid, largely due to low germination in the Procedure 1 mix with 10 ug kg^{-1} clopyralid and moderate EC. This effect was not consistent as other interaction effects such as (EC × clopyralid) and (EC × procedure) were not significant. Pea germination in the high-EC compost in Procedure 1 mixes was not inhibited, despite test media conductivity over 10 dS m^{-1}. This result corroborates data we reported previously in which germination of peas was severely inhibited by only one of the two high-EC composts using Procedure 1, indicating phytotoxicity was not due to a single factor.

In contrast to peas, red clover germination was severely inhibited by the moderate and high-EC composts using Procedure 1 but not when using Proce-

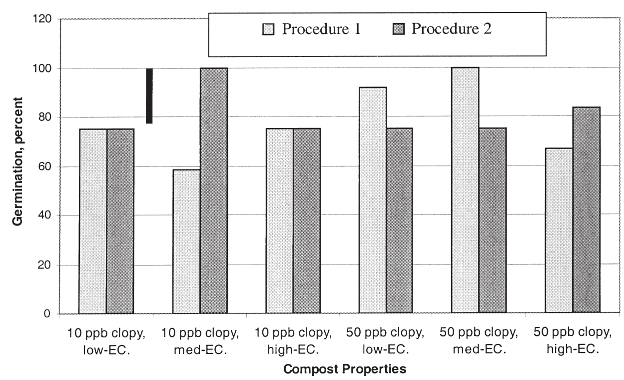

FIGURE 1. Comparison of field pea germination from bioassays using Procedure 1 and Procedure 2. Black bar shows the LSD values for p-0.05 for the procedure-EC-clopyralid interactions

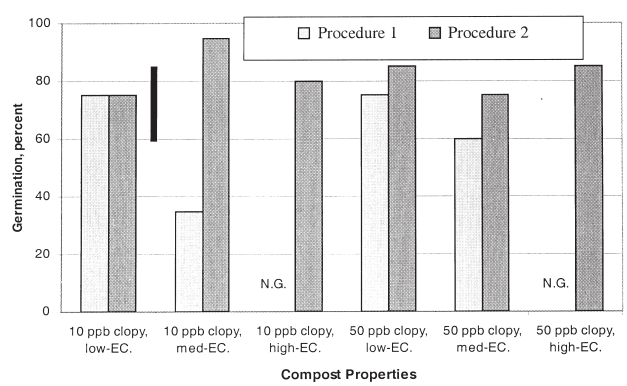

FIGURE 2. Comparison of red clover germination from bioassays using Procedure 1 and Procedure 2. Black bar shows the LSD values for p-0.05 for both the procedure means and the procedure-EC interactions. N.G. = no germination.

dure 2 (Figure 2) similar to previously observed effects (Brinton *et al.*, 2005). Clover germination rate was negatively correlated with EC of both the compost (r^2 = 0.79; $p < 0.01$) and test media (r^2 = 0.69; $p < 0.05$) in Procedure 1 but not in Procedure 2 (Figure 3a). This high degree of correlation of germination with initial compost EC indicates that negative EC effects carry through even after compost dilution, if sufficient diluent is not used. The absence of a correlation between compost EC and germination for Procedure 2 (Fig. 3a) was expected based on the hypothesis that the systematic dilution eliminates differences in compost EC and therefore removes the inhibition, which is also clear from the satisfying cluster of data points for Procedure 2 in Figure 3b.

A critical value for EC effect on germination may be obtained by employing a Cate-Nelson grid for germination vs. EC of bioassay media (Cate and Nelson 1965). With this approach an overlay consisting of 4-quadrants is moved to the point where data in the +/+ quadrants are at a maximum (Figure 3b for Procedure 1). The intersection on the x-axis defines the critical threshold; in this case an EC slightly more than 3.5 dS m^{-1}, in close agreement to published guidelines (Bailey *et al.* 2004, 2005). Both procedure and the interaction (EC × Procedure) had significant effects on germination of clover. Therefore, in view of germination effects, only the Procedure 2 protocol would be suitable for this choice of plants. In earlier work it was observed that the Procedure 2 protocol eliminated all germination differences for four tested plant species, whereas the Procedure 1 protocol caused stunting, chlorosis and germination failure for certain species, most severely for beans and red clover. Beans are commonly used in the popular versions of herbicide bioassays (ODEQ 2003; SPU 2002). In one study, beans used as bioassay plants indicated significantly different thresholds of sensitivity when used in two different years (ODEQ 2003), an effect which is likely attributable to variable and high EC.

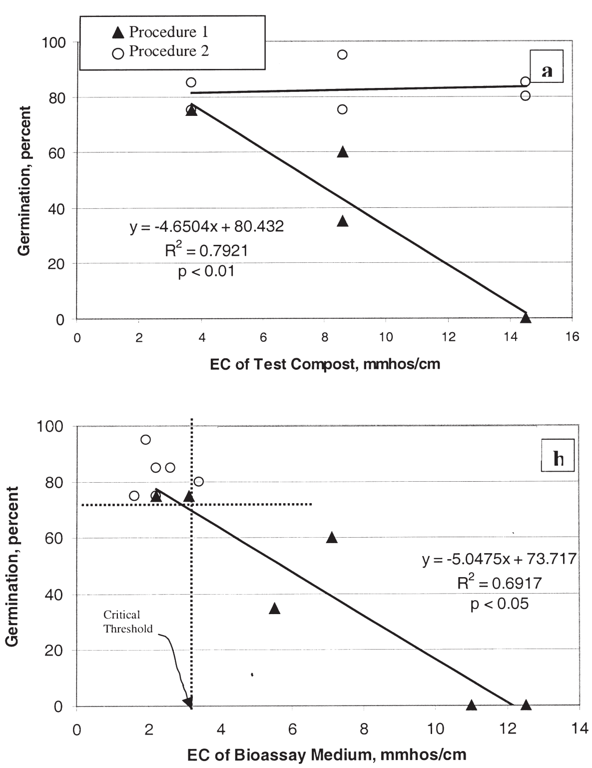

FIGURE 3. Effect of compost EC (a) and bioassay media EC (b) on red clover germination in Procedure 1 and Procedure 2 clopyralid bioassay.

Plant Biomass

Field pea yield depression due to high EC was observed when Procedure 1 was employed at both low and high clopyralid levels, which probably obscured herbicide symptomology (Figure 4). However, for Procedure 2, we observed that the yields of peas increased somewhat with the increasing rates of dilution to accommodate salt content. It was not possible to determine definitively if yield effects indicated for

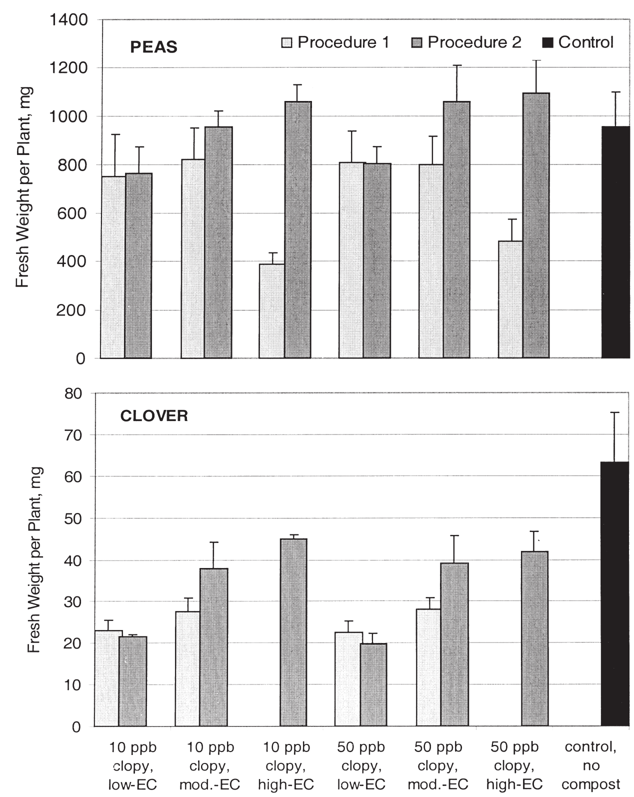

FIGURE 4. Comparison of PROCEDURE 1 and PROCEDURE 2 clopyralid bioassay procedures for Field Peas and Red Clover, on 17-day seedling fresh weights. N.G. = no germination. Error bars indicate one standard deviation. For statistical values of p for comparisons of each variable level, see Table 4.

TABLE 4.
Levels of significance (p-values) from ANOVA for compost-induced effects on bioassay seedling biomass (fresh weight).

	EC	Clopyralid	Proced.	EC x Proced.	EC x Clopy.	Clopy x Proced.	All Three
Field Peas	0.017	0.42 NS	0.005	0.017	0.96 NS	0.68 NS	0.34 NS
Red Clover	0.001	0.65 NS	0.001	0.001	0.80 NS	0.61 NS	0.86 NS

NS = not significant

both procedures resulted from lower clopyralid concentrations, or the composition of the compost, or both. Conversely, while biomass increased steadily using Procedure 2 with increased dilutions of the compost, it is not possible to say if this is a result of dilution of clopyralid or improved plant conditions by reduction of other interfering phytotoxins.

For clover the trends were nearly identical at the low and high clopyralid levels. With Procedure 1 there was no germination under high EC conditions, and therefore no measurable biomass. For Procedure 2, the yield increased proportionally to media dilutions required in that procedure, as previously observed with peas. Since positive visual symptomology of herbicide damage was observed at all levels for red clover, it is possible that greater biomass was partly a result of dilution of herbicide, but most likely only where initial herbicide levels were high. The composts used to prepare the high EC composite scored only 65% relative growth by the *Lepidium sativum* (cress) test (data not shown), which employs a similar salt dilution method. Cress is a brassica and is insensitive to clopyralid but indicates other phytotoxicity factors. Thus, it is not possible to conclude that biomass increased with red clover purely as a result of dilution of herbicide residues. ANOVA analysis of biomass showed that neither procedure distinguished low-clopyralid from high-clopyralid compost based on plant weights (Table 4).

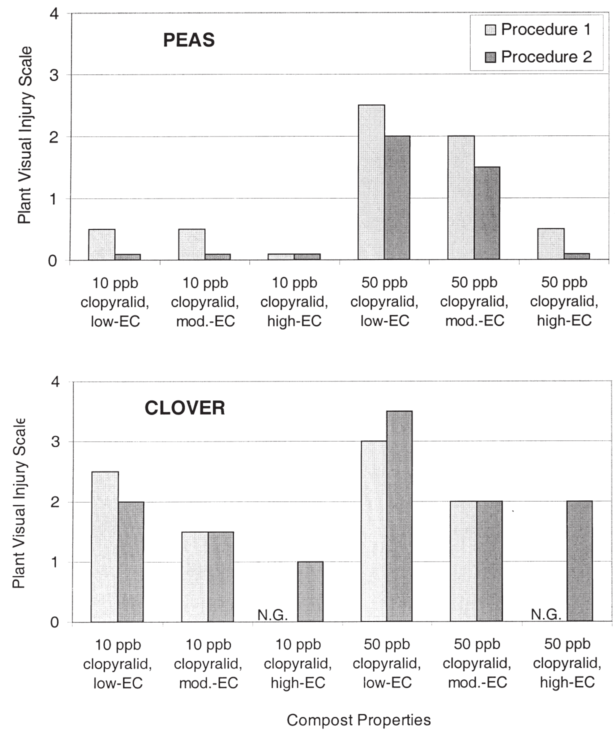

FIGURE 5. Visual injury ranking of Clopyralid-type symptomology observed in 17-day field pea and red clover seedlings grown according to PROCEDURE 1 and PROCEDURE bioassay. N.G. = no germination. See Table 3 for visual injury scale.

Herbicide Visual Injury Symptoms

Procedure 2 indicated no visual symptoms of injury for field peas at all EC levels in low-clopyralid composts (Figure 5). However, with Procedure 1 a slight leaf curl (injury scale = 0.5) for low and medium EC blends was observed but no symptoms for high EC blends. Thus, for Procedure 1, increased EC changed the apparent expression of herbicide symptomology, since the level of clopyralid remained the same throughout. For Procedure 2, the clopyralid concentration in the media declined as the adjustment for EC increased, and therefore it could be expected that appearance of symptomology may be reduced in high EC mixes if clopyralid concentrations were already close to a lower limit of detection.

When high clopyralid composts (50 ug kg^{-1}) were employed, both procedures gave average visual injury ratings of 2, 1 and 0 with peas for low, medium and high EC blends, respectively. The difference in appearance of herbicide symptoms in plants using Procedure 1 was therefore an artifact of the procedure and not an effect based on the actual growing mix, since the clopyralid concentration had remained constant. For Procedure 2, however, the declining symptomology reflects the increased dilution of media to account for EC.

For the 10 ug kg^{-1}clopyralid compost treatment, Procedure 1 indicated phytotoxicity symptomology of 2.5, 1.5 and no-germination (NG) for Red Clover under the three EC levels (low, medium, high) despite the clopyralid concentration being nearly the same in all three. In a previously reported nonherbicide trial, high EC in media resulted in stunting or total-loss of red clover (Brinton *et al*. 2005). Using Procedure 2, the observed plant symptoms for herbicide damage rated 2, 1.5 and 1 for the same three EC levels due to the increasing compost dilution employed by that technique. For the 50 ug kg^{-1} clopyralid composts, Procedure 1 gave a similar result with herbicide damage ratings of 3, 2 and no-germination (NG) at the low, medium and high EC levels. The corresponding effects using Procedure 2 were 3.5, 2 and 2. In this case, Procedure 2 buffered the negative effects of high EC and accurately reflected a measurable presence of herbicide in high EC mixes. Procedure 1 failed with high EC blends due to confounding salt factors, which resulted in complete inhibition of germination. Thus the stated objective of the Procedure 1 approach to elicit a large likelihood of observing clopyralid effects in compost by using high concentrations in the growing media is defeated at the upper end of the range. Both procedures however have greatly reduced sensitivity to detect herbicide presence in high EC composts, but for opposite reasons.

The reliability of the two procedures may be summarized by employing rank-order correlation to evaluate the strength in relationship of visual injury symptoms to measured levels of clopyralid, for all concentrations and blends (Figure 6). For field pea,

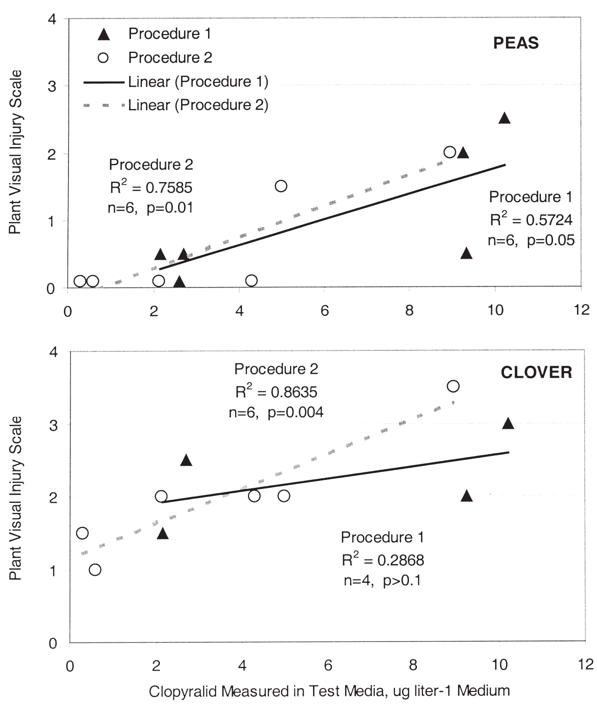

FIGURE 6. Relationship of the observed clopyralid-type damage in field peas and red clover in 17-day seedlings vs. clopyralid measured in the bioassay test media, expressed as ug clopyralid per liter of medium, in the PROCEDURE 1 and PROCEDURE 2 clopyralid bioassay procedures.

both procedures produced similar slopes, and statistically significant p-values, with a more robust r^2 for Procedure 2. This indicated that despite the greater dilutions used in Procedure 2 there was minimal difference in reliability compared to Procedure 1 while eliminating the confounding effects of variable EC levels in the media.

For red clover, a plant more sensitive to clopyralid and EC levels, the differing response between the two procedures was much more pronounced when using rank-order correlation. Procedure 1 failed to substantially discern clopyralid phytotoxicity responses (r^2 values of 0.28, p value >0.10) compared to Procedure 2 (r^2 of 0.86 and p-value of 0.004). Procedure 2 had a steeper slope in this case, whereas some bioassay plants in Procedure 1 suffered total loss due to salt effects (resulting in a loss of 2 pairs of data).

Conclusions

Our findings indicated it is important to factor out high EC in composts used for bioassays by diluting with an inert media to avoid indirect yield loss and germination damage due to salt levels. Procedure 1 used for peas alone provides reliable indication of herbicide residues, but will result in some plant growth impairment independent of herbicide, should salts be very high. The greatest sensitivity and reliability was found when employing red clover and Procedure 2, which accurately discerned low and high herbicide levels at all levels of compost EC. Future work should attempt to identify effects occurring between the medium and high level of EC employed in this study, as a great many composts are likely to fall in that range. While high EC is an obvious phytotoxicity variable that must be controlled, additional effort is also needed to understand the role of pH and other phytotoxicity factors that may be present in composts and compromise bioassays.

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