EFFICACY OF ROOT DIGESTION AND MACERATION IN DETERMINING ABUNDANCE OF ROOT-INHABITING NEMATODES

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ABSTRACT

Alternatives were sought to the standard root staining and plate counting technique for determining abundance of root-inhabiting plant parasitic nematodes, as it is time consuming and laborious. The use of KOH, cellulase, pectinase, EDTA, sodium hypochlorite bleach, root staining or tap water alone or in combination with heating or maceration in a Waring blender was investigated as tools for extracting the root knot (*Meloidogyne*), clover cyst (*Heterodera*) and lesion (*Pratylenchus*) nematodes from white clover (*Trifolium repens*) roots. Abundance of nematodes extracted from, and remaining in, roots after maceration was determined to assess the efficacy of the various treatments. The best method involved staining roots by the standard method followed by a 10 second pulse of blender maceration. For seedlings, extraction of *M. trifoliophila* nematodes by the best maceration method was not significantly different to that of the standard method but macerated samples took ca 6× less time to assess than the standard method.

INTRODUCTION

In most North Island pastures white clover roots are invaded by three genera of nematodes (endoparasites): *Meloidogyne*, *Heterodera* and *Pratylenchus*. It is common for all three genera to be found within the root systems of white clover plants growing in field soil. Unlike many other nematodes it is not possible to get a complete population assessment simply by extracting nematodes from soil as not all life stages are present in soil. For *Meloidogyne* and *Heterodera* only the infective juvenile stage (J2) and males are present in soil with the other juvenile stages (J3 and J4) and live females found only in roots. *Pratylenchus* is a migratory parasite of roots so that all stages can be found in soil but there is always a proportion of the population inside roots, and this can vary across seasons (MacGuidwin 1989). In addition, when assessing nematode control options it is possible that treatments that have an effect on one of the endoparasitic nematode genera may open up niche space for one of the other genera (Yeates et al. 1985) and root counts may well reveal such effects.

A common method used to examine nematodes in plant roots is by staining the roots and squashing or dissecting them on glass plates where they can be counted with the aid of a microscope. When nematode populations become dense, counting and identifying them in roots is often very difficult and time consuming. Inaccuracies can arise for the staining and plate counting method in thick or overlaid roots, in root galls and where nematodes are behind the vascular tissue on the inferior surface of the root.

The aim of this study was to find an efficient method for extraction of root inhabiting nematodes in white clover compared to standard conventional methods of staining and counting.

MATERIALS AND METHODS

The pre-treatment and maceration times of the 16 methods evaluated for extraction of *Meloidogyne trifoliophila* from white clover roots are given in Table 1. The plants were growing in pots of peat:sand mix inoculated with eggs of *M. trifoliophila* as part of the maintenance of a pure culture of this nematode.

Method 1 is the standard method (bleach+staining) where white clover roots were separated from shoots, blotted dry and transferred to 1.5% NaOCl (bleach) for 4 min with frequent agitating. Bleach was made by diluting Janola® (4.2% NaOCl with 4% available Cl) with tap water to the required NaOCl concentration. Roots were then rinsed in running tap water for 30 s followed by soaking in tap water for a further 15 min. Roots were blotted dry and placed in a tube of boiling stain solution (equal amounts of glycerol, lactic acid and distilled water, with 0.05% aniline blue added), boiled for 30–45 s then allowed to cool to room temperature. Finally, stained roots were rinsed in running water and blotted dry then stored in acidified glycerol (equal volume of glycerol and distilled water plus few drops of lactic acid) until assessment.

For maceration roots were blotted dry on tissue and weighed before being cut into 2–5 mm pieces and transferred to a Waring 1 litre glass blender attachment. They were macerated in 100 ml water at high speed for 3×10 s pulses followed by 30 s at low speed. After maceration the blender was thoroughly rinsed with the rinsings pooled into the macerate. The macerated suspension was transferred to a 150 ml plastic beaker and allowed to settle for 4 hours after which the volume was reduced by aspiration to 10 ml. This concentrated nematode and root suspension was transferred to a Doncaster dish (Doncaster 1962) and the nematodes counted.

Several methods were used to treat clover roots before maceration in an attempt to soften the roots and enhance extraction of nematodes from the roots. Methods 4, 5 and 6 were variations of the method developed by Araya & Caswell-Chen (1994) while the bleach methods (7 and 8) were as described by Stetina et al. (1997). Ethylene diamine tetra-acetic acid (EDTA) was used in Method 10 in a similar manner to Stynes (1976). Fresh root weights used were: 0.3 g for Methods 2–6 and 10; 0.2 g for Methods 11–14 and 16; 0.4 g for Methods 8, 9 and 15; and 1 g for Method 7. A comparable weight of roots was used for the standard method as for each of the treatments. The water volume used in maceration was 100 ml except for Method 7 where water was replaced with 60 ml of 0.5% bleach.

To investigate whether the time taken to stain root samples could be reduced, the bleaching (Method 17) or root staining (Method 18) steps were omitted and compared with the standard plate method and Method 16. For this experiment, white clover seedlings were grown in field soil augmented with nematodes from pure culture to ensure roots were invaded by *Meloidogyne*, *Heterodera* and *Pratylenchus* nematodes and five replicate samples assessed by each method. All macerations were carried out as previously described.

Finally, effects of maceration time on nematode extraction were determined by reducing maceration from 3×10 s pulses at high speed (Method 21) to 2 (Method 20) or 1×10 s pulse at high speed (Method 19). In this experiment, the 1 litre blender attachment was replaced with a 100 ml stainless steel blender attachment and initial water volume reduced to 75 ml. Again, five replicate samples were assessed for each method.

Data were analysed by ANOVA using Genstat after log_e transformation. Back-transformed means and standard error of means are presented.

RESULTS

Of the fifteen alternative methods of extracting nematodes from white clover roots, ten (Methods 2–10 and 13) gave reduced numbers of nematodes compared to the standard root staining and glass plate method (Method 1) (Table 1). Of those that gave comparable or greater numbers of nematodes, two yielded large amounts of root debris which slowed counting (Methods 11 and 12), while a third resulted in a high proportion of damaged

or misshapen nematodes (Method 14). Methods 15 and 16 gave the best results both in terms of numbers extracted and integrity of nematodes, although some damage to nematodes due to maceration was still evident.

TABLE 1: Qualitative comparison of maceration methods to extract *Meloidogyne trifoliophila* nematodes from white clover roots grown in culture pots. All macerations carried out in a 1 litre blender attachment.

Method No.	Treatment	Maceration time (and speed)	Comments including number of nematodes relative to Method 1
1	Standard: Bleach + staining	—	Time consuming Difficult to count nematodes in galls
2	1% KOH at 60°C for 30 min	30 s (High)	Small number of J3 + J4 and females
3	Water at 60°C for 4 h	30 s (High)	Small number of J3 + J4 and females
4	Cellulase at 37°C for 36 h	30 s (High)	Small number of all stages Time consuming
5	Pectinase at 37°C for 36 h	30 s (High)	Small number of all stages Time consuming
6	Cellulase+Pectinase at 37°C for 72 h	30 s (High)	Small number of all stages Time consuming
7	0.5% bleach soak for 5 min	20 s (High)	Juvenile stages heads missing Some females left in galls
8	1.5% bleach soak for 4 min	30 s (High)	Small number of all stages
9	Water at 60°C for 4 h	30 s (High) + 3 × 10 s (Low)	Small number of all stages
10	0.07M EDTA at 65°C for 24 h	20 s (High)	Small number of females J3+J4's heads and tails missing
11	Fresh roots	3 × 10 s (High) 30 s (Low)	Increased number of females Increased amount of root debris
12	2% boiled formalin +water	3 × 10 s (High) + 30 s (Low)	Comparable number of all stages Juveniles and females damaged Increased amount of root debris Formalin fumes
13	70% ethanol for 6 days	3 × 10 s (High) + 30 s (Low)	Small number of all stages Nematodes misshapen
14	Ethanol+ glycerol for 7 days	3 × 10 s (High) + 30 s (Low)	Comparable number of all stages Juvenile tails missing or misshapen
15	Bleach + staining	30 s (High) + 3 × 10 s (Low)	Comparable number of all stages
16	Bleach + staining	3 × 10 s (High) + 30 s (Low)	Greater numbers of all stages

Quantitatively, the combination of bleach, root staining and maceration (Method 16) yielded significantly ($P<0.05$) less J3+J4 and female *Meloidogyne* nematodes but significantly more J3+J4 *Heterodera* nematodes, compared to the standard method (Table 2). Omitting either bleaching (Method 17) or staining (Method 18) from the method resulted in significantly ($P<0.05$) poorer extraction of J3+J4 *Meloidogyne* and total nematodes than the standard or full root staining plus maceration procedure. Method 1 extracted the greatest number of *Pratylenchus* but this was not significantly greater ($P>0.05$) than Method 16.

TABLE 2: Back-transformed mean *Meloidogyne*, *Pratylenchus*, *Heterodera* and total nematodes/g root ($\pm$ SEM) from white clover seedlings grown in field soil augmented with nematodes from pure culture. All macerations were carried out in a 1 litre blender attachment with 3×10 s pulses at high speed followed by 30 s at low speed.

Method No. Treatment		<i>Meloidogyne</i>				<i>Pratylenchus</i>
		J2	J3+J4	Female	Male	
1	Standard: Bleach + staining	208 (86)	1367 (254)	1179 (394)	81 (27)	549 (149)
16	Bleach + staining + maceration	328 (135)	854 (159)	268 (89)	125 (42)	350 (95)
17	Staining + maceration	189 (78)	332 (62)	391 (130)	148 (50)	251 (68)
18	Bleach + maceration	35 (14)	181 (34)	253 (84)	112 (38)	201 (55)
		<i>Heterodera</i>			Total nematodes	
		J2	J3+J4	Female		
1	Standard: Bleach + staining	171 (46)	94 (25)	23 (7)	3405 (562)	
16	Bleach + staining + maceration	243 (65)	342 (89)	39 (12)	2730 (450)	
17	Staining + maceration	70 (19)	71 (19)	15 (5)	1376 (227)	
18	Bleach + maceration	143 (38)	251 (65)	28 (9)	1145 (189)	

Reducing maceration time to 10 s (Method 19) in a 100 ml blender attachment resulted in significantly ($P<0.01$) greater extraction of J2 *Meloidogyne* nematodes from roots, compared to the standard method or maceration times of 20 or 30 s (Table 3). There was no significant difference between the number of other stages of *Meloidogyne* extracted using Method 19 and the standard method. The maximum (3×10 s) maceration time resulted in significantly ($P<0.05$) reduced extraction of J3+J4 *Meloidogyne* compared to the standard or Method 19.

TABLE 3: Back-transformed mean *Meloidogyne trifoliophila*/g root (± SEM) extracted from white clover roots growing in culture pots and the time taken to count samples. All macerations carried out in a 100 ml blender attachment at high speed.

Method		<i>Meloidogyne</i> /g root				Time in min.
No.	Treatment	J2	J3+J4	Female	Male	
1	Standard:	17 (5)	792 (137)	216 (62)	9 (4)	38 (13)
	Bleach + staining					
19	Bleach + staining + 1 × 10 s maceration	74 (23)	575 (99)	180 (52)	7 (3)	6 (0.2)
20	Bleach + staining + 2 × 10 s maceration	6 (2)	423 (84)	98 (27)	6 (3)	6 (0.2)
21	Bleach + staining + 3 × 10 s maceration	27 (8)	357 (62)	75 (22)	8 (3)	6 (0.2)

DISCUSSION

This study has shown that using stained roots as a pre-treatment to mechanical maceration was more effective than other pre-treatment methods, and that the combination of bleaching and staining gave better results than either alone. The duration of maceration was also important and the best method (Method 19) required just a single 10 s pulse at high speed to extract comparable quantities of nematodes to the much more time consuming (ca 6× longer) plate counting method. Similarly, Marks & McKenna (1981) found that blender maceration of stained potato roots reduced counting time of *Globodera* nematodes by at least 8×.

The root knot nematode *Meloidogyne* was used in initial work because it is the most difficult to extract from the roots (N.L. Bell, unpubl. data), therefore any technique that could successfully extract *Meloidogyne* sp. should be useful for the majority of other root-inhabiting nematodes.

Many of the pre-maceration treatments tested left the nematodes in a turbid solution, from which it was difficult to count the nematodes. However, the staining and maceration method yielded a reasonably clear counting solution with only small fragments of intact plant tissue. Root staining also allows preservation of nematodes for long periods in acidified glycerol until assessments could be done.

It was of interest to note that more J2 *Meloidogyne trifoliophila* nematodes were extracted by the best maceration method (Method 19) than the standard method. It is unclear whether this was due to the maceration rupturing some female nematodes resulting in vermiform nematodes being released or if the effect illustrated the difficulty in accurately counting J2 *Meloidogyne* in stained roots using plate counting.

Macerated samples contained some nematodes with their tails broken off, which made identification to *Meloidogyne* or *Heterodera* difficult, particularly as the nematodes got to the J3+J4 stages. This may produce differences in the abundance of these genera between macerated and plate-counted roots. The magnitude of this effect has yet to be assessed but it may pose a problem in instances where roots are invaded by both *Meloidogyne* and *Heterodera* nematodes.

The advantages of the root staining and 10 s pulse maceration method developed here are that it extracts comparable numbers of nematodes from the root as the standard method, in addition to being simple, inexpensive and rapid. This allows for greater numbers of samples to be processed in a given time and means that greater replication can be employed when assessing the effects of applied treatments on nematodes (e.g. Sarathchandra et al. 2001).

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