RESEARCH/INVESTIGACIÓN

EXAMINING SUSCEPTIBILITY OF WHITE CLOVER, BUCKWHEAT, BLACK OAT, AND FORAGE RADISH AS A LONG-TERM COVER CROP MIX TO *MELOIDOGYNE INCOGNITA*

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ABSTRACT

Pitiki, M., R. Paudel, J. Mew, and K.-H. Wang. 2024. Examining susceptibility of white clover, buckwheat, black oat, and forage radish as a long-term cover crop mix to *Meloidogyne incognita*. Nematropica 54:41-48.

White clover (*Trifolium repens*) is a popular perennial ground cover for orchard crops in the tropics. However, due to its slow-growing nature, mixed planting of buckwheat (BW, *Fagopyrum esculentum*), black oat (BO, *Avena strigosae*), forage radish (FR, *Raphanus sativus*) with white clover (CL) is needed to help with initial establishment of the ground cover. The objective of this research was to examine the susceptibility of these cover crops to *Meloidogyne incognita*. Two greenhouse trials were conducted comparing the susceptibility of BW, BO, CL, and FR to *M. incognita*. Mustard green (*Brassica juncea*) was included as the susceptible control, and sorghum (*Sorghum bicolor*) was included as the resistant control. White clover had the highest number of females with eggs per gram of root in both trials. BO, BW, and FR had similar number of females without eggs per gram of root as CL and mustard green. The lower inoculation rate in Trial II, 300 *M. incognita* second-stage juveniles/pot, allowed for more distinct segregation of susceptibility of cover crops toward *M. incognita* based on reproductive factor (Rf = final population density/initial population density), which was highest in CL. Despite a low Rf, BW was highly intolerant to *M. incognita* and senesced early. In conclusion, mixed planting of the varieties of BW, BO, CL, and FR tested here are not recommended in soil infested with *M. incognita*.

Key words: Orchard ground covers, plant-parasitic nematodes, resistance, root-knot nematodes, reproductive factor, susceptibility

RESUMEN

Pitiki, M., R. Paudel, J. Mew y K.-H. Wang. 2024. Examinando la susceptibilidad del trébol blanco, trigo sarraceno, avena negra y rábano forrajero como mezcla de cobertura de cultivos a *Meloidogyne incognita*. Nematropica 54:41-48.

El trébol blanco (*Trifolium repens*) es una cubierta vegetal perenne popular para cultivos de huertos en los trópicos. Sin embargo, debido a su crecimiento lento, es necesario realizar una siembra mixta de trigo sarraceno (BW, *Fagopyrum esculentum*), avena negra (BO, *Avena strigosae*), rábano forrajero (FR, *Raphanus sativus*) con trébol blanco (CL) para ayudar con el establecimiento inicial de la cubierta vegetal. El objetivo de esta investigación fue examinar la susceptibilidad de estas cubiertas vegetales a *Meloidogyne incognita*. Se realizaron dos ensayos en invernadero comparando la susceptibilidad de BW, BO, CL y FR a

M. incognita. Se incluyó mostaza verde (*Brassica juncea*) como control susceptible y sorgo (*Sorghum bicolor*) como control resistente. El trébol blanco presentó el mayor número de agallas en las raíces y de hembras de nematodos con huevos por gramo de raíces. Desafortunadamente, BO, BW y FR presentaron un número similar de agallas por gramo de raíz que CL y mostaza verde. Además, FR presentó un número similar de hembras que CL. Aunque BO fue relativamente tolerante a *M. incognita*, tuvo un factor reproductivo (Rf) > 1 dentro de 1 mes después de la inoculación. A pesar de tener un Rf bajo, BW fue altamente intolerante a *M. incognita* y senesció tempranamente. Por lo tanto, no se recomienda la siembra mixta de las variedades de BW, BO, CL y FR probadas en suelos infestados con *M. incognita*.

Palabras clave: Cubiertas vegetales de huertos, nematodos fitoparásitos, resistencia, nematodos de agalla en las raíces, factor reproductivo, susceptibilidad

INTRODUCTION

Cover cropping has long been recommended for soil health improvement (Clark, 2007). While short-term cover crops have been studied frequently in Hawaii, the impact of perennial cover crops on soil health needs more investigation. The use of perennial cover crops could play an even bigger role in managing soil health in orchards if due to lack of disturbance to soil over time (Schlautman *et al*., 2021). Perennial cover crops could provide long-term ground cover in perennial tree orchards with living roots to support beneficial soil microorganisms (Yang *et al*., 2019). Moreover, long-term cover crops have been shown to increase yields, save on nitrogen costs over time, and lead to a more profitable system (Clark, 2007). It is important to know the susceptibility of cover crops to plant-parasitic nematodes to prevent the build up of nematode densities over time (Fuller *et al*., 2008). In the tropical climates of Hawaii, only a few perennial cover crops are ideal in terms of rate of establishment and low risk of weediness. Perennial peanut (*Arachis pintoi*) is a commonly used perennial ground cover in orchards in Hawaii, but it takes more than eight months to establish (Radovich *et al*., 2009), and availability of seed in the USA is scarce in recent years. Thus, efforts to find an alternative to perennial peanut is needed. Hooks *et al*. (2007) compared three clovers including a biennial (yellow sweet clover, *Melilotus officinalis*), and two perennial clovers (white clover, *Trifolium repens*, and strawberry clover, *Trifolium fragiferum*) that can grow in the warm climate in Hawaii. Yellow sweet clover fully established within 2.5 months of growth, but it continued to grow like a weed and outcompeted the subsequent broccoli (*Brassica oleracea*) crop. It is important to have fast canopy closure of these ground covers so as to outcompete weeds. The canopy of white clover established faster than strawberry clover, but still took more than six months to fully cover the soil (K.-H. Wang, personal observation), which will not serve well as a ground cover if part of the soil remains bare during this time. One approach to overcome the slow establishment of white clover is by mix planting white clover with fast-growing short-term cover crops to: i) keep the ground covered while white clover is establishing, ii) provide some shade, which is favorable for white clover, and iii) allow for the short-term cover crops to die back as white clover establishes.

Three cover crops that have been shown to be good companion crops with white clover in Hawaii are buckwheat (*Fagopyrum esculentum*), black oat (*Avena strigosa*), and forage radish (*Raphanus sativus*) (Wang *et al*., 2023). This cover crop mix allows rapid ground coverage within a few weeks. Nonetheless, white clover has notoriously been reported to be susceptible to *Meloidogyne incognita*. Windham and Pederson (1991) reported that susceptible white clover will decline two to three years after establishment if *M. incognita* is not managed. Susceptibility of white clover to *M. incognita* does vary among genotypes (Windham and Pederson, 1991; Van Den Bosch and Mercer, 1996). Among the white clover varieties, 'New Zealand' white clover is most commonly grown in Hawaii as it is heat and drought tolerant and is well adapted to locations where slugs are a problem (Ogle and St. John, 2008).

The objective of this research was to determine the susceptibility of 'New Zealand' white clover as well as the host status of its companion plants used in mixed cover cropping that are well adapted to Hawaii (buckwheat, forage radish, and black oat) to *M. incognita.*

MATERIALS AND METHODS

Nematode inoculum

The *M. incognita* race 1 culture originated from Poamoho Experiment Station, University of Hawaii and was maintained on coleus (*Coleus scutellarioides*) on an isolated bench in a greenhouse. Plants were watered daily using an automated sprinkler irrigation system and fertilized monthly with 16-16-16 Miracle-Gro fertilizer (Miracle-Gro, Marysville, OH, USA). Nematodes were extracted from the roots by agitating the roots in 0.6% sodium hypochlorite manually for 4 min (Hussey and Barker, 1973) followed by a centrifugal sugar flotation method (Jenkins, 1964). The extracted eggs were incubated in a Baermann tray (Rodríguez-Kabana and Pope, 1981) for 1 week to collect second-stage juveniles (J2) for nematode inoculation.

Greenhouse experiment

Two greenhouse pot trials were conducted on raised benches during the summer of 2022 at Pope Lab Greenhouse, University of Hawaii at Manoa. Varieties of cover crops tested in both trials were 'New Zealand' white clover (CL), 'Soil Saver' black oat (BO), 'Common' buckwheat (BW), and 'Sod Buster' forage radish (FR). In addition, 'NX-D-61' sorghum (SG, *Sorghum bicolor*) and 'Hirayama' mustard green (M, kai choi, *Brassica juncea*) were included as the negative and positive control, respectively. Ten seeds of each test crop were seeded in a 12-cm-diam. clay pot filled with 500 cm^3 sterile sand: soil mix (1:1 v/v) and grown for 2 weeks prior to nematode inoculation. Each test crop was replicated in four times for each trial. In Trial I, each pot was inoculated with 600 J2, whereas in Trial II, each pot was inoculated with 300 J2 delivered through 3 ml of water per pot at 3 insertion points. The high rate of inoculation in Trial I lead to rapid senesce of some of the cover crops tested, thus a lower rate was used in Trial II. Trial I was conducted from May 2 to May 30, 2022, whereas Trial II was conducted from June 1 to June 29, 2022, from nematode inoculation to termination. Both trials were arranged in a completely randomized design. Plants were watered as needed throughout the duration of the experiment. Average day length was 14.3 hr, and average maximum and minimum temperature of a day were 31.2 and 21.4°C, respectively.

At the termination of each trial, roots from each pot were harvested, washed, weighed, and 0.3 g roots were subsampled for acid fuchsin staining to facilitate visualization of development of *M. incognita* inside the roots (Byrd *et al*., 1983). Stained roots were observed under a dissecting microscope (Leica Microsystem Company, Wetzlar, Germany) to quantify numbers of second-third-, and fourth-stage juveniles (J2, J3, and J4), females in galls with and without eggs (Fig. 1), and galls without females. Total number of *M. incognita* per gram of root or per pot, and reproductive factor (Rf) were calculated. Rf refers to the final total number of *M. incognita* (regardless of life stage) in the roots per pot divided by the inoculum density.

Statistical analysis

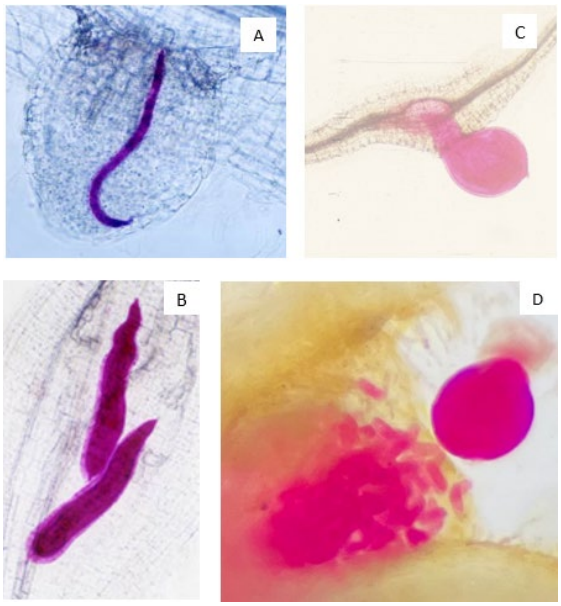

Fig.1. Stages of *Meloidogyne incognita*: A) second-stage juvenile (J2); B) fourth-stage juvenile (J4); C) female without egg masses; and D) female with egg masses in cover crop roots stained with acid fuchsin one month after nematode inoculation. Picture credit: Marisol Davila and Roshan Paudel

Data from each trial were subjected to one-way analysis of variance (ANOVA) separately. Numbers of *M. incognita* per gram of root at different stages of development, root weight per pot, and Rf from both trials were first checked for

normality using Proc Univariate in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Skewed data were $\log_{10}(x+1)$ transformed prior to ANOVA. Means were separated using Waller-Duncan k-ratio (k=100) t-test whenever appropriate. Only true means were presented.

RESULTS

Nematode numbers per gram of roots

Among different life stages, number of *M. incognita* females with or without eggs were significantly different among the crops tested in both trials ($P \leq 0.05$, Table 1). Data from both trials revealed that CL had the highest number of egg-laying females, significantly higher than the other cover crops ($P \leq 0.05$; Table 1), except for SG in Trial II, although only minimal numbers of egg-laying females were found on SG in Trial II (Table 1). In Trial I, BW and FR also started to form egg-laying females, but the numbers were lower than CL ($P \leq 0.05$). Buckwheat, CL, and FR supported similar number of females without eggs as the susceptible control, M, in Trial I ($P > 0.05$), whereas BO supported minimal number of females without eggs as the resistant control, SG ($P > 0.05$). In Trial II, CL, FR, and BO had higher number of females without egg masses than BW ($P \leq 0.05$). The number of galls without females was not different among the cover crops in Trial I ($P > 0.05$), but CL had the highest number of galls without females than the other cover crops in Trial II ($P \leq 0.05$), similar to that in the susceptible control (M). When combining all life stages of *M. incognita* together, no differences among cover crops were detected in Trial I (Table 1), but a significantly higher number of *M. incognita* were found in CL than SG in Trial II ($P \leq 0.05$).

Root weight

Buckwheat senesced early and only had minimal root biomass remaining at the termination of both trials. Though not significantly different from CL, M, and FR in Trial I (Fig. 2A), BW had the lowest root biomass among the cover crops in Trial II (Fig. 2B). In fact, the root systems of CL, BW, and FR were severely stunted or senescing at 1 month after *M. incognita* inoculation. As expected, SG had the healthiest root system with the highest root weight per pot in both trials ($P \leq 0.05$, Fig. 2A, B). On the other hand, BO had a relatively vibrant root system, with higher root weight than CL, BW, and FR in both trials ($P \leq 0.05$, Fig. 2A, B).

Total M. incognita *per pot and reproductive factor*

By extrapolating the total number of all life stages of *M. incognita* inside the roots per pot, no significant difference was detected among crops

Table 1. Different life stages of *Meloidogyne incognita* per gram root on different crop hosts in Trial I and Trial II at 1-month after inoculation.

Crop	J2[x]	J3/J4[y]	Female (- eggs)	Female (+ eggs)	Galls	Total (all stages combined)
			Trial I			
Black oat	1 a[z]	36 a	3 b	0 b	64 a	104 a
Buckwheat	0 a	16 a	41 ab	2 b	66 a	125 a
White clover	0 a	7 a	21 ab	14 a	21 a	63 a
Mustard green	0 a	2 a	36 ab	0 b	11 a	49 a
Forage radish	0 a	2 a	120 a	1 b	15 a	139 a
Sorghum	1 a	8 a	1 b	0 b	1 a	11 a
			Trial II			
Black oat	0 a	0 a	10 a	0 b	5 bc	15 ab
Buckwheat	0 a	40 a	0 b	0 b	0 c	40 ab
White clover	0 a	2 a	56 a	16 a	45 a	120 a
Mustard green	1 a	0 a	5 ab	0 b	7 ab	12 ab
Forage radish	0 a	1 a	27 a	0 b	6 bc	34 ab
Sorghum	0 a	1 a	5 ab	1 ab	1 bc	8 b

[x]J2 = second-stage juveniles

[y]J3 = third-stage juveniles, J4 = fourth-stage juveniles

[z]In each trial, means (n=4) in a column followed by different letter(s) are different based on Waller-Duncan k-ratio (k=100) t-test.

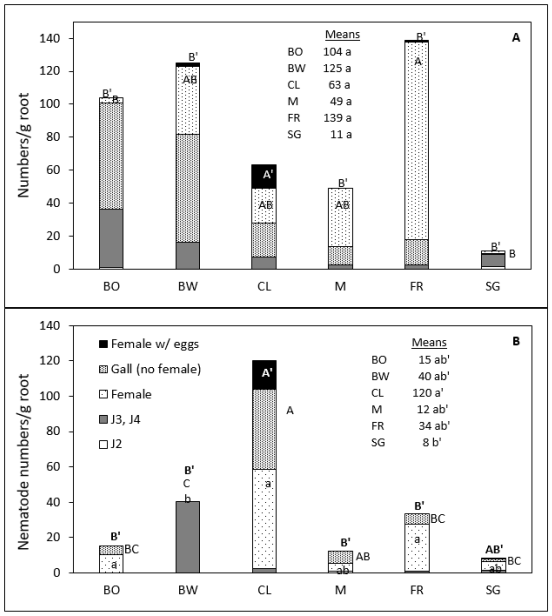

Fig. 2. Root weight (g) of cover crop per pot at termination of the experiment in Trial I (A) and Trial II (B). Bars (n =4) with the same letter(s) are not different based on Waller-Duncan k-ratio (k=100) t-test. BO = black oat, BW = buckwheat, CL = white clover, M = mustard green, FR = forage radish, and SG = sorghum.

tested in Trial I (Fig. 3A). However, when a lower inoculum density was used in Trial II, CL and BO supported a higher number of total *M. incognita* in the roots per pot than BW ($P \leq 0.05$; Fig. 3B). Similar results were observed when comparing Rf of *M. incognita* where no difference was detected in Trial I (Fig. 4A), but CL had higher Rf than BW in Trial II ($P \leq 0.05$, Fig. 4B). When higher inoculum was used in Trial I, all except BW resulted in Rf >1, but at the lower inoculum level in Trial II (Fig. 4B), only CL supported Rf >1.

DISCUSSION

Orchard farmers in Hawaii take advantage of CL as a ground cover because it can fix nitrogen and adapt to a wide range of soil types and pH from 5.5 to 7 (Smith and Valenzuela, 2002). Once established, its dense, spreading stolon and root system makes it excellent for erosion control and weed suppression (Smith and Valenzuela, 2002). However, the risk of CL in increasing root-knot nematode population densities had been overlooked. Results from this study confirmed that 'New Zealand' white clover is very susceptible to *M. incognita* and should be avoided in soil infested with this nematode. It is unfortunate that the three companion cover crops (BW, BO, and FR) previously shown to assist CL establishment in the field were also relatively susceptible to *M. incognita* though to a lesser degree than CL.

The lower *M. incognita* inoculation rate in Trial II than Trial I was to avoid rapid senescence of the test plants for better comparison of nematode susceptibility. Buckwheat as well as CL were very susceptible to *M. incognita*, which led to premature death or highly stunted plants within one month after inoculation. Reducing the inoculation level from 600 to 300 nematodes per pot allowed more segregation in number of females without eggs, total *M. incognita* infection per pot, and Rf in Trial II, which was not detected in Trial I.

While 'New Zealand' CL allowed *M. incognita* to start producing egg masses within one

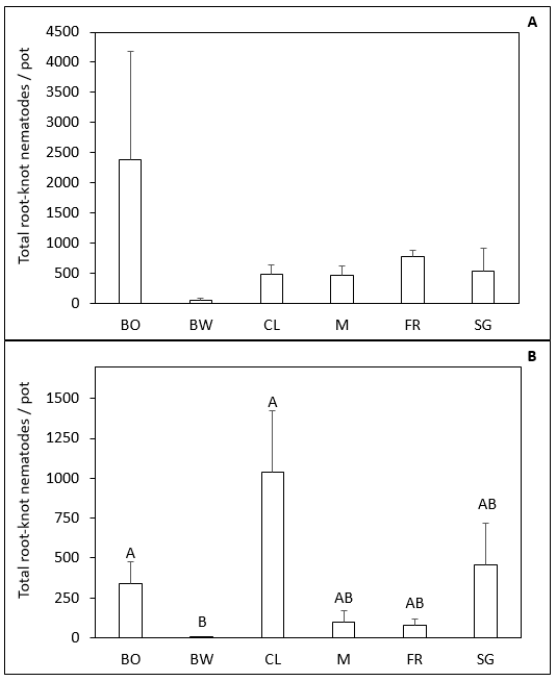

Fig. 3. Total number of *Meloidogyne incognita* per pot for each cover crop in Trial I (A) and Trial II (B). Means (n = 4) followed by the same letter(s) are not different according to Waller-Duncan k-ratio (k=100) t-test. BO = black oat, BW = buckwheat, CL = white clover, M = mustard green, FR = forage radish, and SG = sorghum.

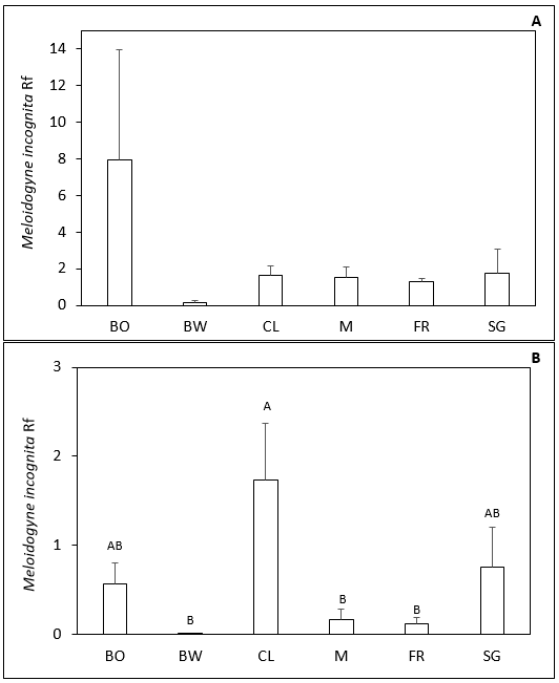

Fig. 4. Reproductive factor (Rf) of *Meloidogyne incognita* on black oat (BO), buckwheat (BW), white clover (CL), mustard green (M), forage radish (FR), and sorghum (SG) at 1 month after nematode inoculation for Trial I (A) and Trial II (B). Means (n= 4) followed by the same letter(s) are not different according to Waller-Duncan k-ratio (k=100) t-test.

month after nematode inoculation, 'Common' BW started to senesce during this period without going into the egg-laying stage in Trial I and stopped at J3/J4 stages in Trial II. Sipes and Arakaki (1997) demonstrated that BW planted prior to a taro crop enhanced root-knot nematode populations dramatically. Gardner and Caswell-Chen (1994) also showed that BW was susceptible to *Meloidogyne javanica*. However, our results showed that the susceptibility of BW to *M. incognita* might be less of a concern since BW will senesce before *M. incognita* can reproduce as shown in the Rf value of < 0.2 in both trials.

The susceptibility of BO and FR to *M. incognita* would also be less of a concern if the initial inoculum is low (≤ 300 J2/500 cm^3 soil), with Rf < 1 at termination of the trial. However, at a high inoculum level (*e.g.*, 600 J2/500 cm^3 soil), 'Soil Saver' BO had numerically higher total density of all stages of *M. incognita* in the roots per pot and Rf, though this was not statistically different from CL, M, FR, and SG. The higher density of *M. incognita* in the roots and Rf were due, in part, to the relatively high amount of root biomass generated by BO. This result is similar to the findings of Marquez *et al*. (2022) who reported that BO was susceptible to *M. incognita* based on egg index (number of egg masses/root). The current study on the developmental stages of *M. incognita* showed that BO supported slower development into egg-laying females than CL. Despite being susceptible to *M. incognita*, BO was considered tolerant of *M. incognita* infection based on the definition of nematode tolerance by Trudgill (1991) because, despite its high Rf, BO showed no sign of stunted growth with high root mass production. Nematode tolerance is defined as the capacity of a plant host to withstand plant-parasitic nematode infection and reproduction without significant damage (Trudgill, 1991).

'Sodbuster' FR had the highest number of females per gram of roots in Trial I but not in Trial II where the inoculum level was 300 J2/500 cm^3 soil. Waisen *et al*. (2019) reported that 'Sodbuster' was a poor host of *M. incognita* compared to tomato based on egg density/g root though it was susceptible to *Rotylenchulus reniformis*. However, in both trials of this study, the Rf value of 'Sodbuster' was <1, indicative of a poor host, and it was less susceptible to *M. incognita* than CL.

Hajihassani *et al*. (2020) determined that root-gall index is not a reliable indicator of the susceptibility or resistance of root-knot nematode but rather egg index is in a greenhouse setting. The current study examined different stages of *M. incognita* inside the roots, providing additional information such as how quickly the females developed on a host plant or if nematode development is arrested at certain stages. We also noticed that some root galls did not have any life stages of *M. incognita* nor egg masses associated with them, indicative that fully matured females and their egg masses might slough off during the root washing and staining process, which is unavoidable and should not be ignored. Future research using root staining to evaluate plants with high susceptibility and low tolerance to *M. incognita* should record various developmental stages of this nematode including galls with and without the female intact.

Overall, in this study, *M. incognita* developed the fastest on CL. The susceptible control, 'Hirayama' mustard turned out to be less susceptible than CL. However, our negative control 'NX-D-61' sorghum was the most resistant to *M.*

incognita infection and development. Farmers can preplant 'NX-D-61' sorghum and incorporate it into the soil to suppress *M. incognita* in an infested field (Paudel *et al*., 2021) prior to establishing CL as a long-term cover crop.

In conclusion, CL should not be recommended as ground cover if soil is infested with *M. incognita*. Mix planting CL with 'Common' BW, 'Sodbuster' FR, and 'Soil Saver' BO will not reduce *M. incognita* reproduction. Thus, farmers should determine the plant-parasitic nematodes present in their soil before planting these cover crops.

ACKNOWLEDGMENTS

The authors thank Koolau Seed Supplies and University of Hawaii Seed Lab for providing seeds for this study. This project is supported by NRCS CIG Hawaii NR1992510002G001 and NR2192510002G002, NIFA OREI (HAW09705-G), CTAHR Hatch, Multistate (NE2140), Plan of Work (HAW9048-H, 9034-R and POW 16-964), and WSARE graduate student grant GW20-212.

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Received: *17/VII/2023*

Recibido:

Accepted for publication: *5/I/2024*

Aceptado para publicación: