Selection for resistance against root pathogens in a pea composite cross

Jens C. Knudsen[1], John Larsen[2*], Lars Bødker[2], Bjarne Jørnsgård[3] and Birgit Jensen[2,4]

[1]Toft Plant Breeding, Tønderingvej 14, DK-7870 Roslev, Denmark

[2]Department of Integrated Pest Management, Aarhus University, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark

[3]Department of Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Højbakkegård Alle 30, DK-2630 Taastrup, Denmark

[4]Department of Plant Biology, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

[*]Author of correspondence: John.Larsen[a]agrsci.dk

Abstract

The possibility of improving resistance in pea against the root pathogen *Aphanomyces euteiches* using composite cross as a breeding and selection method was examined. In order to maintain acceptable agricultural features and high yield 6 out of the 8 parental varieties in the present composite-cross were commercially grown varieties. Populations of the composite cross were grown up to five generations with selection pressure in soil heavily infested with pea root pathogens or without selection pressure on soil free of pea root pathogens. Yield of populations of the F_9 and F_{10} generations of the composite cross grown with selection pressure was on average 35% higher than that of the population obtained without selection pressure as well as the average yield of the 8 parentals of the composite cross, which were of similar magnitude. In healthy soil the yield was overall higher than in the pathogen-infested soil, but yield did not differ between the populations from the composite cross with and without selection pressure, which were also similar to the average yield of the 8 different parentals. Recombinant inbred lines (RILs) randomly selected from the F_{10} population with selection pressure developed 23% less root rot than the corresponding F_{10} population without selection pressure, when grown in field soil heavily infested with pea root pathogens. Surprisingly, greenhouse pot experiments with pure cultures of the pea root pathogen *A. euteiches* resulted in higher root disease, in RILs from populations with selection pressure than from corresponding RILs without selection pressure. Problems related to greenhouse screening for resistance is discussed as well as the possibilities of using composite cross as a method to improve resistance against root diseases in grain legumes.

Keywords: organic farming, root pathogen, plant breeding, legumes

Introduction

In organic farming, soya and other protein sources play an important part in the production of pigs and poultry. To meet the requirement for protein in a feed self sufficient-organic farm with a high proportion of monogastric animals, the proportion of grain legumes in rotation should be at least 30% to 50% (ref). Grain legumes, e.g. pea (*Pisum sativum*), faba beans (*Vicia faba*) and lupins (*Lupinus* sp.) can complement cereals in animal feed. Besides being a valuable protein source, these grain legumes benefit the farming system via biological nitrogen fixation and by being a break-crop for cereal diseases. Therefore limitations, which reduce the maximum ratio of grain legumes crops in the organic rotation as well as their productivity, are direct limitations for the expansion of organic farming (ref).

The biggest obstacle for an increased proportion of grain legumes in the organic rotation is presently diseases, which are accumulated in the system over time, especially soil and seed borne pathogens (ref). Pea root rot caused by *Aphanomyces euteiches*, is often regarded as the most destructive pathogen of pea (*Pisum sativum*) in areas with humid climates (Kraft and Pfleger, 2001), including Souhtern Scandinavia (Persson et al, 1997). In areas with longest tradition for pea growing, 10-20% of the fields are not suitable for pea production due to high levels of natural infestation of pea root pathogens (ref). It is expected that at least 20 years is necessary before pea growing can be taken up again in these natural infested fields (ref). This persistence of legume pathogens is therefore a threat in organic farming systems because the biological fixation of atmospheric nitrogen is a fundamental process for maintaining soil fertility.

World wide different breeding methods have been employed to obtain plant resistance against root rot pathogens (refs), however as several genes are involved in

resistance against *A. euteiches* it is difficult to obtain resistant varieties (refs). Various breeding methods are used when introducing resistance genes into highly adapted material (refs). Methods involve backcrossing, where defined genes are transferred, recurrent selection involving repeated cycles of inter-mating and selection often used in pyramiding genes in out breeding species and composite crosses used in self pollinating cereals (). In this project the "composite cross" method developed by Suneson (1956), will be evaluated as a tool for selecting breeding lines with improved resistance. In this method the F_1 progeny from crosses of different plant genotypes with agronomic important features are bulked and subsequently exposed to selection in successive natural cropping environments. This breeding method seems to be particularly well fitted for low input systems such as organic farming (Phillips and Wolfe, 2005; Murphy et al, 2005).

The objective of the present study was to examine the possibility of using "composite cross" as a breeding- and selection method to achieve improved resistance in pea against the root pathogens focusing on *Aphanomyces euteiches*.

Materials and methods

Description of the pea composite cross

A composite cross was created with 8 different pea cultivars (Table 1) differing in resistance to the root pathogens *A. euteiches* and *F. oxysporum* and also differing in other agronomic characteristics following the crossing scheme in Table 2. Crosses were carried out in the greenhouse during the winters 1993 and 1994 and F1 seed grown till F2 during the same period. It was attempted that each F2 population consisted of at least 400 seeds. The F2 were grown in the field and harvested bulk for each population. Each population was divided in two, and grown for the next 3 to 5

generations under two different selection regimes. One populations was grown under heavy selection pressure of soil borne pathogens in a field cropped continuously with pea for 7 years. The other population was grown on land free of pea soil borne pathogens. F_7 populations were harvested in the field in 1998 and stored. Stored seed were sown in plots in 2002. From each population 150 F_7 plants were taken at random, forming the recombinant inbred lines (RILs) for the further studies. Remaining part of plots were harvested bulk for each population. RIL's of the two final composite lines were multiplied in rows in the field in 2003, a season characterised by severe attacks of Mycosphaerella that affected seed quality. In the winter 2003/04 all populations from 2002 and the eight parentals were multiplied under disease free conditions in the southern hemisphere to establish seed populations of equal germination capacity for trials 2004. Trials 2005 was sown with seed harvested in trials 2004, representing a further cycle of selection.

Field trials

Yield

In 2004 and 2005 three identical trials were sown on land with varying levels of infestation with soil borne root pathogens. Each trial consisted of the eight parentals, the 14 populations and 3 further commercial control varieties sown in 3 replicates in an alpha-design. Sowing density was 65 germinating seeds per m^2 sown with an Oyord drill. Trials were treated with pre- and post emergence herbicides to control weeds and when necessary with insecticides as well. No fungicides were used. The disease severity was controlled using plants in the border plots, which were scored for root rot.

Evaluation of tolerance to soil borne pathogens in RILs

From each of the composite cross populations 150 RILs lines were selected at random in F_7. These lines together with the parental lines in 2004 were sown in small plots on heavily infested land. Each plot consisted of one 1-m row with seeds sown with a pneumatic precission drill to space plants 8 cm apart given 12 plants per plot. The trial had two replicates of each RIL and the set of parentals was included seven times. On the 19th and the 26 th of July the rows were scored by a scale 0 to 5 for yellowing of above ground parts. The degree of yellowing was taken as a measure of attack of soil borne pathogens on below ground plant parts. RIL's were again tested in the dirty plot field in 2005 using the same design as in 2004. DSI was measured three times during the growing season; 24th June, 3rd and 18th of July.

Green house pot experiments

Screening RILs for *A. euteiches* susceptibility

RILs from 124 lines from (F?) populations obtained with and without selection pressure were screened for susceptibility towards *A. euteiches* Dreschler (ATCC 2016). The experiment was performed with a randomized block design each with 31 RILs from the two populations over a four-day period. Each RIL had two replicates.

Sandy loam soil from Research Centre Flakkebjerg was partially sterilised by irradiation (10 kGy, 10MeV electron beam) and mixed with quartz sand obtaining a ratio of 1:3 soil:sand (w/w). Basal nutrients were mixed into the soil in the following amount (mg kg^{-1}): xxxxx.

Oospore-based inoculum of *Aphanomyces euteiches* Dreschler (ATCC 2016 84), was produced by growing the fungus in oatmeal broth (0.5% oatmeal in

demineralised water) at 20°C in the dark for eight weeks. Thereafter, the suspension with mycelium and oospores was homogenised for two minutes in a blender and filtered twice through gauze. The suspension was washed with a sterile dilute salt solution (Fuller and Jaworski, 1987) three times by centrifugation at 3000 rpm for four min. and the oospores were counted in a haemocytometer. Finally, the suspension containing oospores allowed to dry on 100 g quartz sand, and thereafter mixed homogeneously into the soil:sand mix resulting in a concentration of approximately 400 oospores g^{-1} soil. A similar amount of quartz sand without oospores was added to the treatments without *A. euteiches.* Seeds were surface sterilised in 1.5% NaOCl for eight minutes, washed three times in demineralised water, pre-germinated for three days, and sown at a depth of three cm with 14 seeds per 1.25 l pot (12 cm diameter, 14 cm height), containing 1600 g soil:sand mix, both with and without fungal inoculum. At sowing, 2 ml of a dense *Rhizobium leguminosorum* (Risø strain 18a) culture was added to each pea seed. *Rhizobium* was cultured in sterile yeast mannitol broth (g l^{-1}): $K_2HPO_4 \times 3H_2O$ (0.66), $MgSO_4 \times 7H_2O$ (0.20), NaCl (0.10), D-Mannitol (10.0) yeast extract (0.40); and pH was set to 8.0.

Pea seedlings were thinned to ten per pot after five days. Plants were maintained in a greenhouse November 2003. Temperature and light settings were 20 °C and 16 hours light / 24 hours throughout the experiment. Natural daylight was supplemented with a photosynthetic active radiation of 150 µmole m^{-2} s^{-1} provided by Osram daylight lamps. The pots were placed in a temperature-regulated container providing a constant soil temperature of 20°C. Each pot was watered to 95% field capacity at least every second day.

Plants were harvested three weeks after sowing. At harvest, plants were gently removed from the soil, washed and visually examined for disease severity of the root

(discoloration) by scoring percentage area of the respective plant parts with symptoms. The shoot was cut off just above the cotyledons, dried (80°C for 24 h) and weighed.

Screening RILs for *F. oxysporum* susceptibility

RILs from 150 lines from (F?) populations obtained with and without selection pressure were screened for susceptibility towards *F. oxysporum* ? Race 1 (isolate etc). The experiment was performed with a randomized block design each with 36-37 RILs from the two populations each day over a four-day period. Each RIL had two replicates each with five plants in individual planting holes.

Inoculum of *F. oxysporum* was produced on Czapek Dox Broth (35 g l^{-1}) with a CDAZ solution with the following nutrients (mg l^{-1}): $CuSO4 \times 5H_2O$ (0.22), MnCl2 × 4H2O (1), ZnCl2 (1), Ca(NO3)2 ×4 H2O (0.1), (NH4)6 Mo7O24 (0.2). Five 1x1 cm agar blocks from a 2-weeks old *F. oxysporum* culture on potato dextrose agar with novobiocin was transferred to a flask the Czapek Dox Broth which were incubated at room temperature (approx. 20 °C) in darkness for five days on a vertical rotary shaker (92 rpm) after which spores were harvested and inoculation suspensions with 10^6 spores ml^{-1} were produced.

Seeds were surface sterilised in 1.5% NaOCl for eight minutes, washed three times in demineralised water, pre-germinated for three days. Seeds from each RILs were sown in five separate planting holes in the trays and each tray consisted of 7 x 5 holes of which six rows were sown with six different RILs and one row with a positive control with the highly susceptible pea variety Julia. Each planting hole contained approx. 100 ml sterile vermiculite.

Plants were maintained in a greenhouse in November 2005 where temperature and light settings were 20 °C and 16 hours light / 24 hours throughout the experiment.

Natural daylight was supplemented with a photosynthetic active radiation of 150 µmole m^{-2} s^{-1} provided by Osram daylight lamps. Each tray was placed in a separate trayholder and watered every twice a week or when needed. When the plants were two weeks old their roots were trimmed by cutting approx. 1/3 of the root system and subsequently the roots were dipped in a spore suspension of *F. oxysporum* for 30 minutes. After additional 4 weeks all plants were scored for disease using a disease index based on percent wilting of the shoot of the five plants from each RIL.

Statistics

Multifactor analysis of variance, using General Linear Model, were used to analyse data, using SAS 8e (SAS Institute Inc.1999)

Results

Field experiments

Yield

Yield in 2004 and 2005 in plots with heavy root pathogen infestation levels obtained from the F_9 and F_{10} seed generation, respectively, of the composite cross population with selection pressure was on average 34.5 % higher than the composite cross population without selection pressure and the average of the 8 composite cross parentals (Figure 2). Yield from the plots with intermediate root pathogen infestation and from plots with healthy soil did not differ between two composite cross populations and the parentals (Figure 2). Average yield was highest in healthy soil in both years, except in 2004 where the average yields from the plot with intermediate root pathogen infestation was similar to that of healthy soil. In 2005 however, yield

from plots with intermediate root pathogen infestation was intermediate; in between yield from plots with heavy root pathogen infestation and that obtained from plots with healthy soil (Figure 2).

Disease index

The average DSI based on measurements of yellowing of the shoot obtained from plots grown at the three different levels of root pathogen infestation increased with increasing levels of infestation (Figure 3). In soil with heavy pathogen infestation, DSI was lowest in the composite cross population obtained with selection pressure and the parentals, and furthermore the DSI of the composite cross population obtained without selection had a lower DSI than the average of the 8 parentals (Figure 3). In healthy soil no difference was found between the three different populations. In soil with intermediate levels of pathogen infestation, the DSI of the two composite cross populations with and without selection pressure was similar, but lower than that of the average of the 8 parentals (Figure 3).

Field screening of RILs in a dirty plot

The average score for RILs originating from the population grown under selection pressure was lower than for that grown without selection pressure, although this difference was only significant in 2005 (Fig 4), where the DSI from RILs with selection was 23% lower than that of RILs without selection (Figure 4).

Greenhouse experiments

Screening of RILs against *A. euteiches* and *F. oxysporum*

The average score of RILs screened for *A. euteiches* susceptibility was 15.7% higher in RILs originating from the population grown under selection pressure than that of RILs grown without selection pressure (Figure 5), which also coincided with a lower shoot dry weight of RILs originating from the population grown under selection pressure than that of RILs grown without selection pressure (data not shown). The average score of RILs screened for *F. oxysporum* susceptibility was 11.7% higher in RILs originating from the population grown under selection pressure than that of RILs grown without selection pressure, however this difference was not significant (Figure 6).

Discussion

To our knowledge this is the first report on a pea composite cross breeding for *A. euteiches* resistance. Our findings that the composite cross developed with selection pressure gave lower disease development and higher yield is similar to the results obtained with soy bean composite crosses in relation to Phytophthora root rot and soy bean cyst nematodes (Hartwig et al 1985; Degago and Cavines, 1987).

Composite cross populations can provide dynamic gene pools, which may be usefull in low-input and /or organic agriculture with unpredictable stress conditions caused by pests and pathogens (Phillips and Wolfe, 2005), but selection against other agronomic important traits needs to be considered. In the present study the pea composite cross, obtained with selection pressure, performed similar as the parentals in uninfested soil in terms of yield.

In barley it has been suggested that 15 generations of natural selection is needed to develop populations with improved agronomic fitness (Suneson, 1956). In the present pea composite cross improved resistance was achieved already after four

generations. However, in the fifth generation the composite cross population did not increase yield. Hence, it would be interesting to follow how more selection cycles would effect the composite cross populations in terms of both disease resistance and other agronomic traits. Results from Degago and Caviness (1987) indicate that the bulk breeding method for disease resistance in soybean is more effective when there is constant year-to-year selection pressure. In the present study the root rot levels was overall higher in 2005 than in 2004, which may explain this difference between years.

Different screening techniques of resistance to root diseases in cool season food legumes has been reviewed by Infantino et al (2006), who emphasized the importance of protocol standardization. Despite of high level of standardization used in our protocols we obtained contrasting results from screening RILs for root disease resistance in the “dirty plot” in the field and in the greenhouse screening. Similarly, Pilet-Nayel et al (2005) reported low correlation between field and greenhouse screening of *A. euteiches* resistance, but also good correlation between field and greenhouse screening for A. euteiches resistance has been reported (Moussart et al, 2001). In our study, the *A. euteiches* isolate used for the greenhouse screening was a laboratory pet, but another isolate of *A. euteiches* originating from the “dirty plot” used in the field screening, behaved similar to the laboratory pet isolate (data not shown).

Simulation of natural environmental conditions is difficult especially if not using field soil in the greenhouse tests. One of the main arguments of using greenhouse screening for specific pathogens is to avoid interfering effects from other soil biota, which are interacting with the pathogen and its host. However, the reason for low correlation between field and greenhouse studies may very well rely on such interactions in the field as *A. euteiches* is sharing the root enviroment with other root

inhabiting fungi such as arbuscular mycorrhizal fungi, which has been shown to reduce different disease measures of *A. euteiches* both in the lab (Larsen and Bødker, 2001; Thygesen et al, 2004) and in the field (Bødker et al, 2002). Furthermore, Thygesen et al (2004) showed that one AM fungus induced tolerance in the pea against root rot caused by *A. euteiches*, whereas another AM fungus had no effect. Another, important difference between field and greenhouse screening is the soil temperature. In most greenhouse studies a soil temperature around 20 ºC is most often used why the screening period can be reduced to 3-4 weeks, whereas the soil temperature in many pea growing areas in the pea growing period is between 5-10 ºC, calling for controlled experiments on the influence of soil temperature when screening for resistance.

Recently molecular markers linked to resistance genes in pea against *A. euteiches* have been identified (Pilet-Nayel 2002, Pilet-Nayel, 2005), which makes marker assisted selection possible and as well as development of varieties with multiple disease resistance (Infantino et al, 2006). Furthermore, progress in the understanding of the specificity of soil borne root pathogens of grain legumes is also vital for future breeding programmes (Wicker et al, 2001; Levenfors et al, 2003 Jensen et al, submitted).

Our results indicate that multiplying segregating generations under the selection pressure from the natural soil pathogen population in the dirty plot will select for increased tolerance/resistance. However, the composite cross which is a combined crossing and selection method is time consuming and seems not to be useful in the selection for resistance against specific pathogens. The method might be useful as a future breeding method for different traits including stress tolerance or as suggested by Murphy et al (2005) to obtain genetic variation as a mean for buffering

environmental fluctuations and maintaining important agronomic traits in low-input and organic agriculture.

Acknowledgements

Anne Pia Larsen, Lis Henriksen, Steen Meier and Heidi Schultz are thanked for excellent technical support. This work was supported by a grant from The Danish Research Centre for Organic Farming (DARCOF), research program DARCOF II.

References

Bødker L, Kjøller R, Kristensen K, Rosendahl S. 2002. Interactions between indigenous arbuscular mycorrhizal fungi and *Aphanomyces euteiches* in field-grown pea. Mycorrhiza 12: 7-12.

Ceccarelli, S.S. 1996. Adaptation to low/high input cultivation. Euphytica 92:203–214.

Corte, H.R., Ramalhol, M.A.P., Goncalves, F.M.A., and Abreu, A.D.F.B. 2002. Natural selection for grain yield in dry bean populations bred by the bulk method. Euphytica 123:387–393.

Degago, Y. and Caviness, C.E. (1987). Seed yield of soybean bulk populations grown for 10 to 18 years in two environments. Crop Science 27:207–210.

337 Hartwig, E.E., Kilen, T.C., Young, L.D., and Edwards, C.J.J. (1982). Effects of
338 natural selection in segregating soybean populations exposed to phytophthora rot or
339 soybean cyst nematodes. Crop Science 22:588–590.
340
341 Infantino A, Kharrat, M., Riccioni, L., Coyne, C.J., McPhee, K.E., Grünwald, N.J.
342 (2006). Screening techniques and sources of resistance to root diseases in cool season
343 food legumes Euphytica 147: 201–221
344
345 Kraft JM and Pfleger FL (2001) Compendium of pea diseases and pests. In: Kraft JM
346 and Pfleger FL (eds) The Disease Compendium Series of the American
347 Phytopathological Society. The American Phytopathological Society, St. Paul, MN,
348 USA
349
350 Larsen J & Bødker L. 2001. Interactions between pea root-inhabiting fungi examined
351 using signature fatty acids. *New Phytologist* 149: 487-493.
352
353 Levenfors JP, Wikström M, Persson L and Gerhardson B. 2003. Pathogenicity of
354 *Aphanomyces* spp. from different leguminous crops in Sweden. European Journal of
355 Plant Pathology 109: 535-543.
356
357 Moussart A, Wicker E, Duparque M Rouxel F 2001. development of an efficient
358 screening test for pra resistance to Aphanomyces euteiches. Pages 272-273 in: Proc.
359 4th Eur. Conf. Grain Legumes, Cracow Poland AEP (ed), Paris, France
360

Murphy K, Lammer D, Lyon S, Carter B, and Jones SS. 2005. Breeding for organic and low-input farming systems: An evolutionary–participatory breeding method for inbred cereal grains. Renewable Agriculture and Food Systems: 20: 48–55.

Persson L, Bødker L & Larsson-Wikström M (1997). Prevalence and pathogenicity of root and foot rot of peas in Southern Scandinavia. Plant Disease 81 (171-174).

Phillips SL and Wolfe MS. 2005. Evolutionary plant breeding for low input systems. Journal of Agricultural Science 143, 245–254

Pilet-Nayel, M.L., F.J. Muehlbauer, R.J. McGee, J.M. Kraft, A.Baranger & C.J. Coyne, 2002. Quantitative trait loci for partial resistance to Aphanomyces root rot in pea. Theor Appl Genet 106: 28–39.

Pilet-Nayel, M.L., F.J. Muehlbauer, J.M. Kraft, R.J. McGee, A. Baranger & C.J. Coyne, 2005. Consistent QTLs in pea for partial resistance to *Aphanomyces euteiches* isolates from the United States and France. Phytopathology 95: 1287–1293.

Suneson, C.A. 1956. An evolutionary plant breeding method. Agronomy Journal 48: 188–191

Thygesen K, Larsen J and Bødker L. 2004. Arbuscular mycorrhizal fungi reduce development of pea root-rot caused by Aphanomyces euteiches using oospores as pathogen inoculum. *European Journal of Plant Pathology* 110: 411-419.

Wicker E, Hullé M and Rouxel F. 2001. Pathogenic characteristics of isolates of *Aphanomyces euteiches* from pea in France. Plant Pathology 50: 433-442.

Figure legends

Figure 1. Description of pea composite cross

Figure 2. Yield of composite cross populations with and without selection pressure and average yield of parental varieties in soil with different levels of root pathogen infestation in 2004 and 2005.

Figure 3. Root rot disease index (based on levels of yellowing of the shoot) of composite cross populations with and without selection pressure and average disease index of parental varieties in soil with different levels of root pathogen infestation in 2005.

Figure 4. Frequency of recombinant inbred lines with different levels of root rot (based on levels of yellowing of the shoot) from composite cross populations with and without selection pressure grown in soil heavily infested with *A. euteiches* in 2004 and 2005.

Figure 5. Frequency of recombinant inbred lines with different levels of root rot (based on levels of root discolouring) from composite cross populations with and without selection pressure tested in a greenhouse pot experiment artificially infested with *A. euteiches*.

411 **Figure 6**. Frequency of recombinant inbred lines with different levels of wilt (based
412 on levels of wilting of the shoot) from composite cross populations with and without
413 selection pressure tested in a greenhouse pot experiment artificially infested with *F.*
414 *oxysporum*.

415

Table 1. Parental varieties of the pea composite cross and their known disease resistance against *Aphanomyces euteiches* root rot and *Fusaium oxysporum* wilt and other agronomic traits

Variety	Cotyledon	Leaf	Wilt resistance	Stem length	Aphanomyces
Loto	Yellow	Afila	+	Short, weak straw	Susceptible
86-638	Green	Normal	(+)	Short, weak straw	Tolerance in USA
Montana	Yellow	Afila	+	Short, weak straw	Susceptible
Capella	Yellow	Afila	-	Short, medium	Tolerance in Sweden
Solara	Green	Afila	+	Short, weak straw	Susceptible
LD89-2-33	Yellow	Afila	-	Short, weak straw	Susceptible
Accord	Green	Afila	+	Medium, strong	Limited tolerance
Julia	Yellow	Afila	-	Short, medium	Susceptible

416

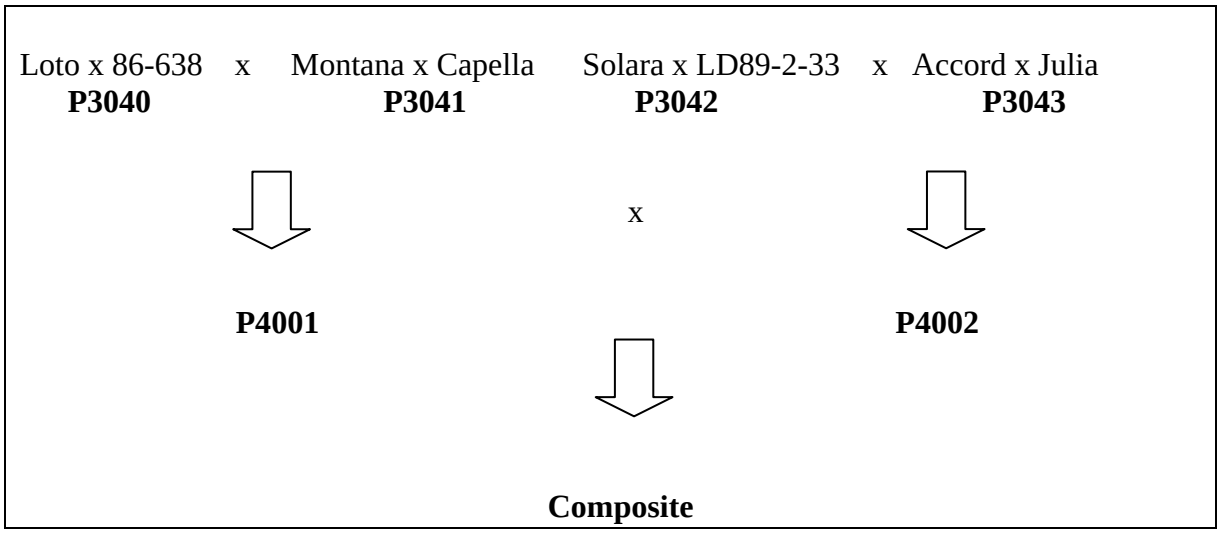
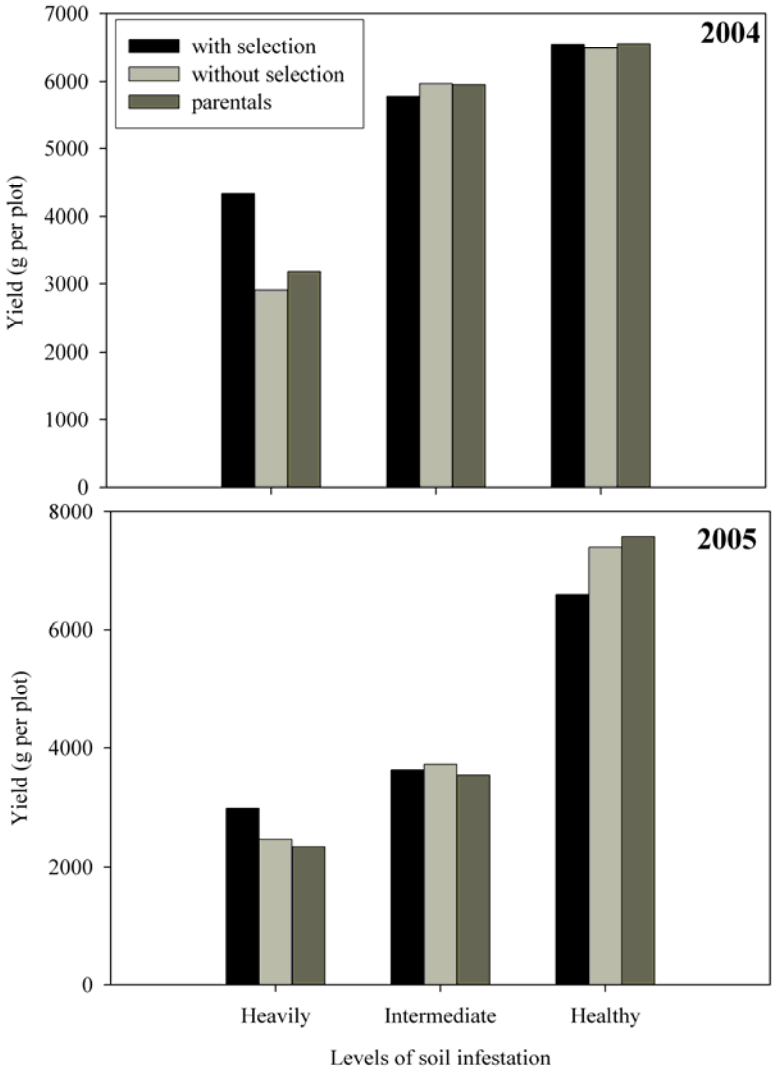
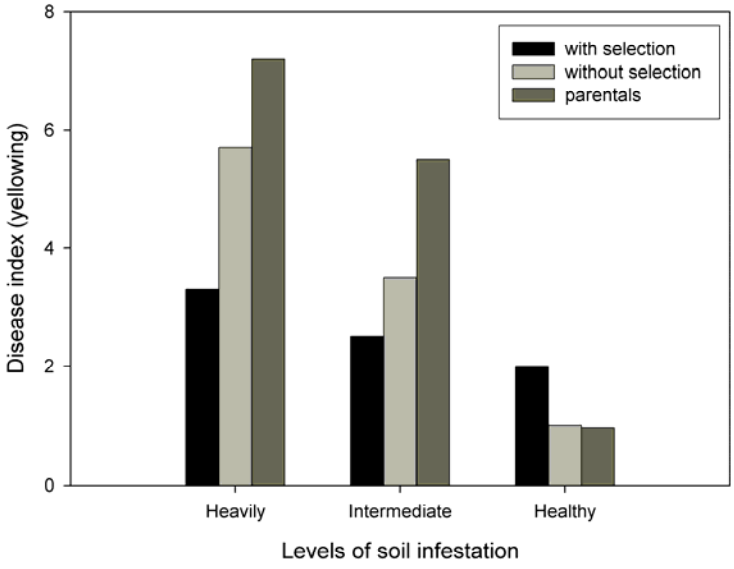

Loto x 86-638 x Montana x Capella Solara x LD89-2-33 x Accord x Julia
P3040 P3041 P3042 P3043
x
P4001
P4002
Composite

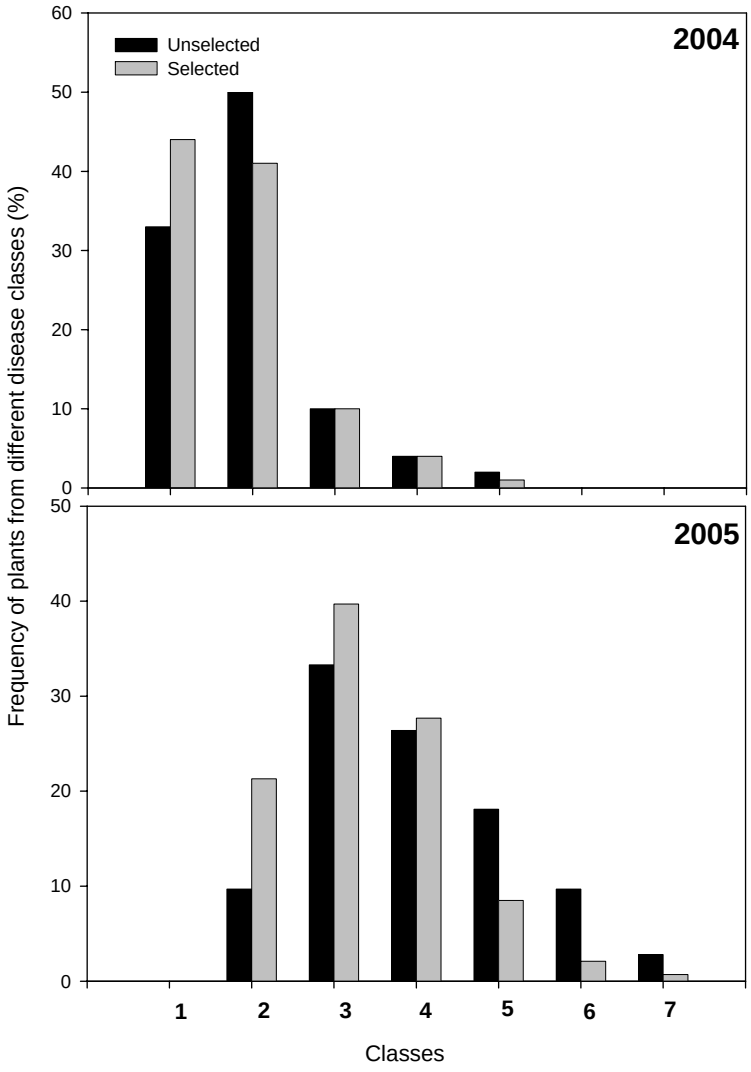
Figure 1



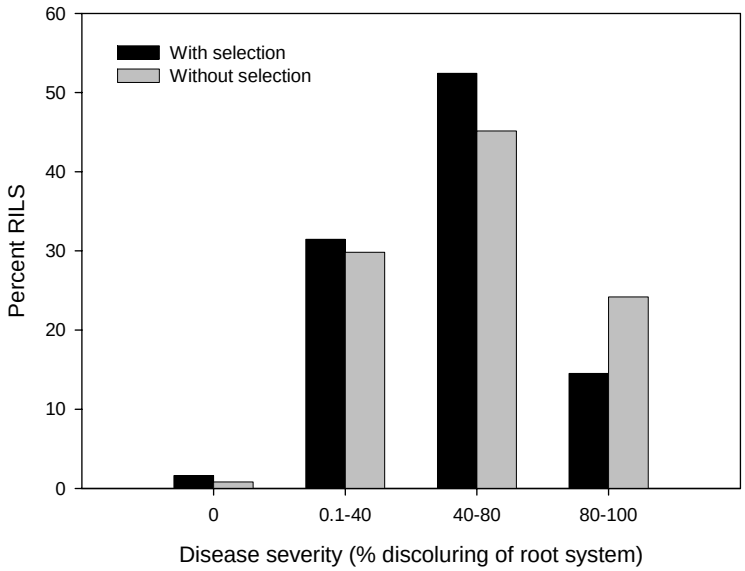
434
435
436 Figure 2



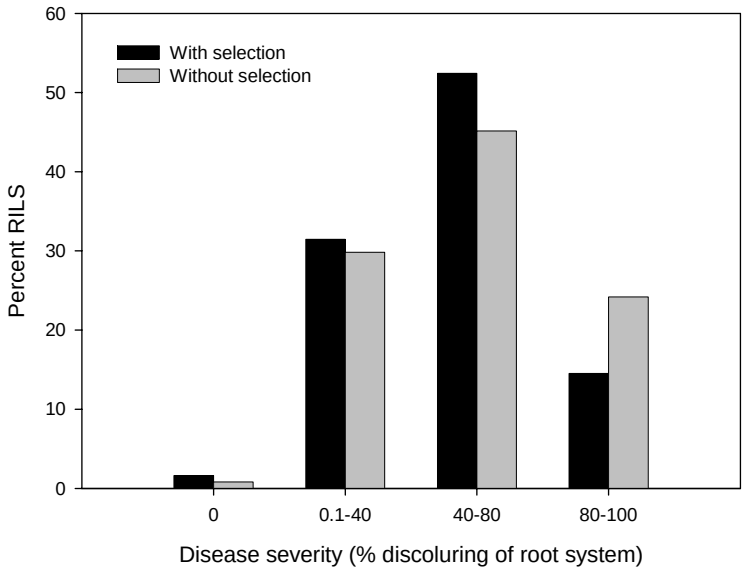
437
438 Figure 3



439
440 Figure 4



441
442
443 Figure 5



444
445 Figure 6