TESTA WP5
Task 5.2:
Comparison of detection methods for *Ditylenchus* in alfalafa and faba bean seed lots and method validation

GEVES
Expertise & Performance

Testa

Background

- **Stem and bulb nematode**

- Caused by *Ditylenchus dipsaci*
- Hosts: wide range of crops (including *Alfalfa* and faba bean)
- Worldwide distribution
- Symptoms on plant:

  swelling and distortion of aerial parts,
  necrosis or decaying at the base of the stems

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Background

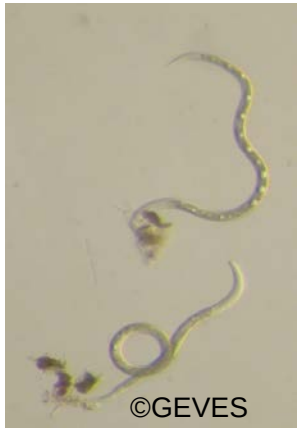
- **Sanitary control.**

- Transmitted by seeds, seed dust, soil and weeds
- Quarantine pathogen on alfalfa seeds in Europe → Regulation

Detection threshold at 0 *D. dipsaci*

Alfalafa	Faba bean
Nematodes on seeds and dust	Nematodes under the seed coat
D. dipsaci	*D. Dipsaci* & *D.gigas*
Many different others nematodes	Less others nematodes
Mainly stage J4 present	All stages present (juveniles, adults)

Background



- **Stem and bulb nematode**

- 30 biological races of *D. dipsaci* described

- A giant race, distinguished from *D. dipsaci*

  → New species named *D. gigas* (*Volvas et al.* 2011)

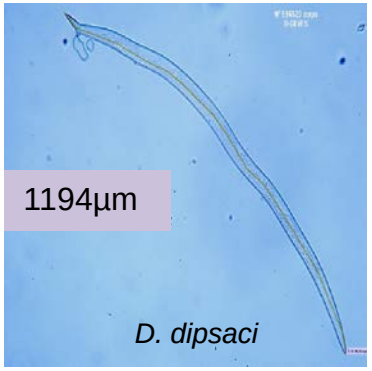
- Molecular methods have been recently developed to confirm the *Ditylenchus* species.
  - Esquibet *et al.,* 2003; Kerkoud *et al.,* 2007; Volvas *et al.,* 2011.

Identification

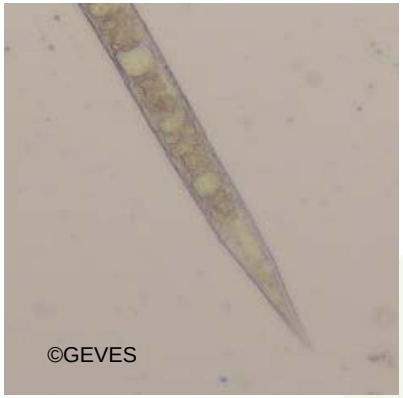
- Morphological criteria difference

Criteria	*D. Dipsaci* (OEPP bulletin 38, 2008)	*D. gigas* (Volvas et al., 2011)
Stylet length (µm)	**10-12µm**	**10-12µm**
Number of lateral lines	**4**	**4**
Body length (µm)	**1000-1300µm**	**1380-1950µm**
Body width	**36-41µm**	**34—63µm**

Body size

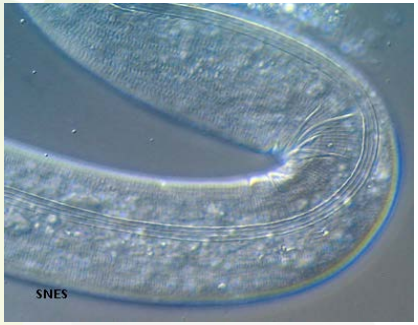


Shape of tail



Shape of lip

Number of line



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Aim & Objectives

- **Aim**
  - To harmonize and validate at international level a detection method of *D. dipsaci* and *D. gigas*.

- **Objectives:**
  - To compare performance of the existing different biological and molecular protocols
  - To validate a method that enable the detection of the *D. dipsaci* and *D.gigas* : **performance characteristics and interlaboratory test**
  - Propose it as an official ISTA and EPPO protocol.

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Comparison of methods

	Decantation method	Filtration method
Reference	NIAB : 013 STNEM beans v5	ANSES : MOA13 part A
Sample size (g) faba bean	500	200
Sample size (g) alfalfa	Not tested	70
Mousseline	Yes dirty samples	Using a paper in the deep of the sieve
Soaking	Overnight (17 hours min)	Overnight (24 hours min)
Sieving	No, leave 4h water standing, pour off the top liquid,	250µm and 20µm sieve,
Examination	keep 100 ml in Petri dish, x25	on the remaining liquid x60
Identification	Morphological. In case of doubt →PCR on a pool of nematodes to confirm *D. gigas* (Wood primers)	Morphological. In case of doubt →PCR on individual nematodes no difference *D. dipsaci*, *D.gigas*.(Kerkoud primers)
Counting	semi quantitative: light (1-15), medium (15-50), heavy (50-500), very heavy contamination (>500)	No, presence/absence only

Comparison of methods

Prestest on alfalfa and faba bean

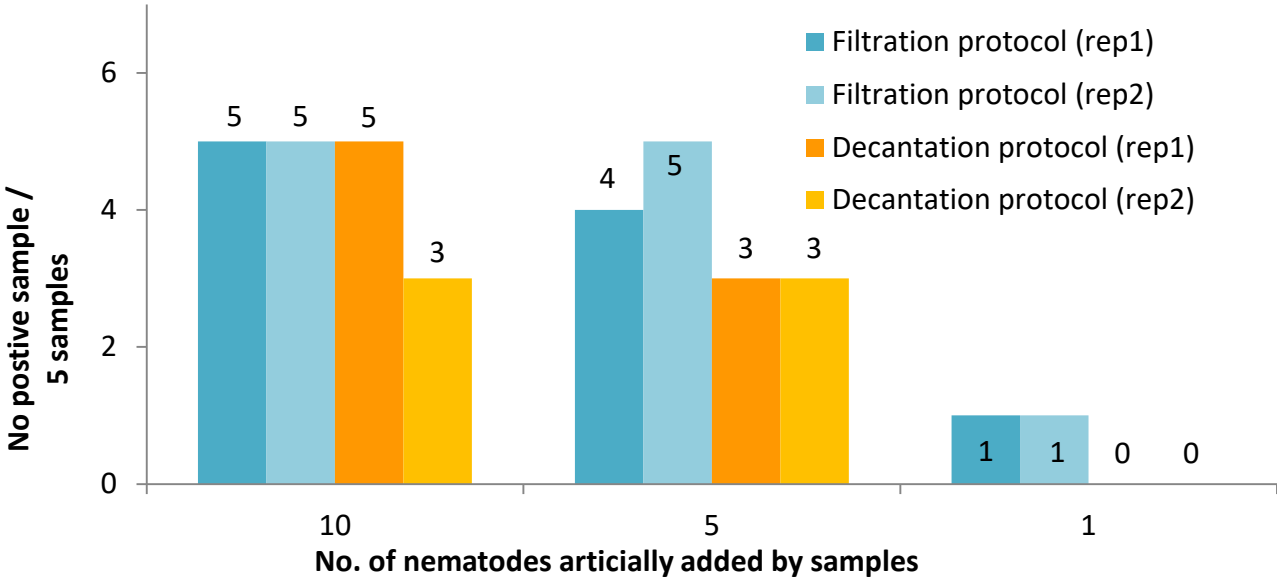
	Filtration protocol (Sieving)		Decantation protocol (bottom)		Decantation protocol (Supernatant)	
Seed lots	Nb positive/ nb samples	Couting	Nb positive/ nb samples	Estimation	Nb positive/ nb samples	Estimation
Alfaalfa 1	5/5	843	5/5	50 to 500	5/5	1 à 15
Alfaalfa 2	5/5	1204	5/5	> 500	5/5	50 à 500
Alfaalfa 3	5/5	1404	5/5	>500	5/5	50 à 500
Faba bean 1	5/5	10190	5/5	> 500	5/5	> 500
Faba bean 2	4/5	454	4/5	50 to 500	4/5	15 to 50
Faba bean 3	5/5	124	4/5	15 to 50	5/5	15 to 50

False negative

Limit of detection

Filtration protocol
High capacity of detection in low infected seed lots

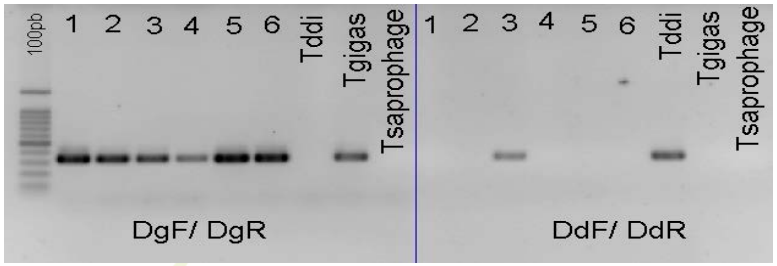
Decantation protocol
limit of detection higher than 10 nematodes



Comparison of methods

Primers availables

Primers	*Ditylenchus dipsaci*		*Ditylenchus gigas*	
Kerkoud *et al* 2007	DdpS1 (F)	rDNA2 (R)	DdpS2 (F)	rDNA2 (R)
Esquibet *et al* 2003	H05 (F)	H06 (R)	D09 (F)	D10 (R)
Wood *et al* 2013	Dd (F)	Dd (R)	Dg (F)	Dg (R)

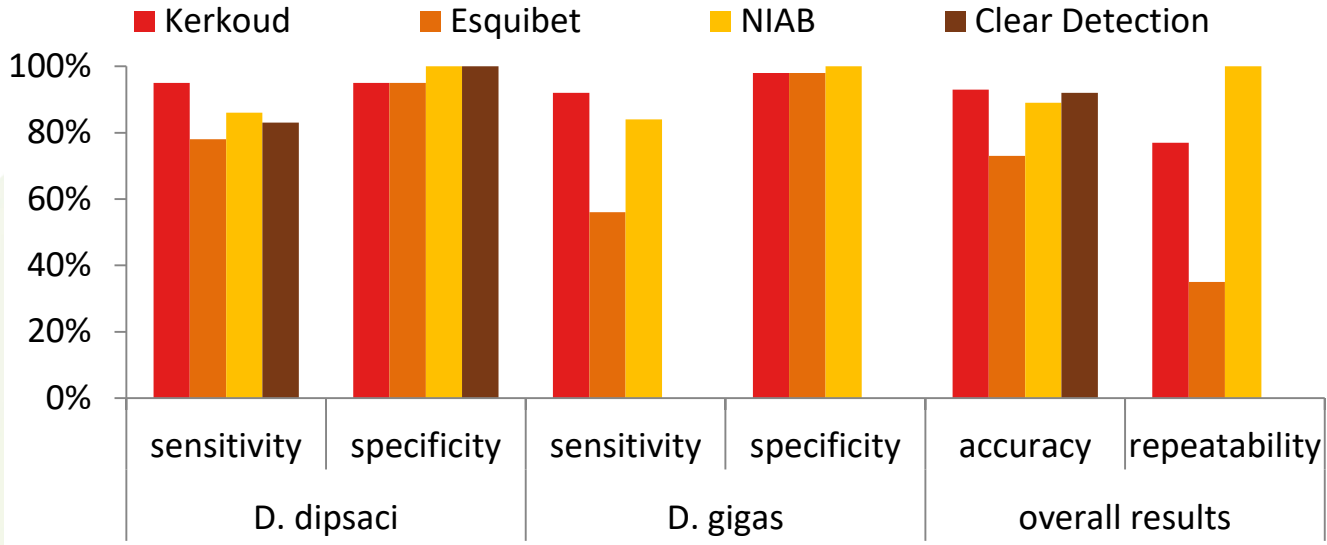


Obtained results

Kerkoud primers
best results
89 % accuracy

Wood primers
allowing identification of both species in mixed samples.

Accuracy and repeatability calculated for the four evaluated methods



Interlaboratory test

- **8 laboratories, filtration method + PCR**
- **3 alfalfa and 3 faba bean seed lots (healthy, medium, high)**
- **Homogeneity test**
  - Done on 3 alfalfa and 3 faba ben seed lots provided by the seed industry
  - 10 samples were analyzed per seed lots.

Qualitative analysis of results obtained after the homogeneity test

	Seed lots	Nb positive samples/total
Alfalfa	Lot A	0/10
	Lot B	10/10
	Lot C	10/10
Faba bean	Lot D	0/10
	Lot E	9/10
	Lot F	8/10

Result proved the homogeneity for seed lot A, B, C, and F

Seed lot E and F were not homogenous

The seed lots were used for the validation test

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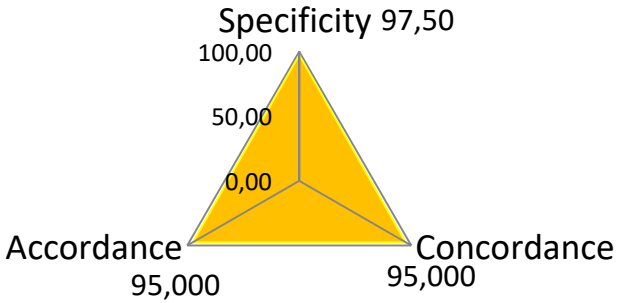
Validation test

Results

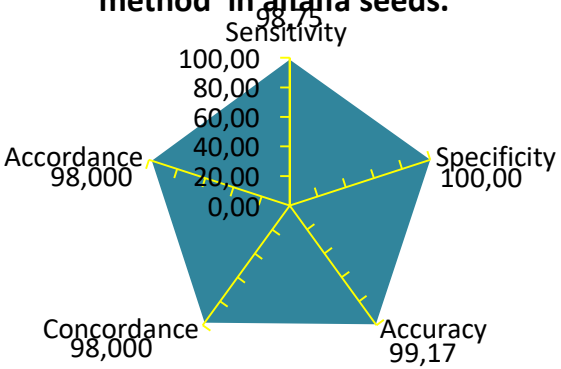
Conform to expected results

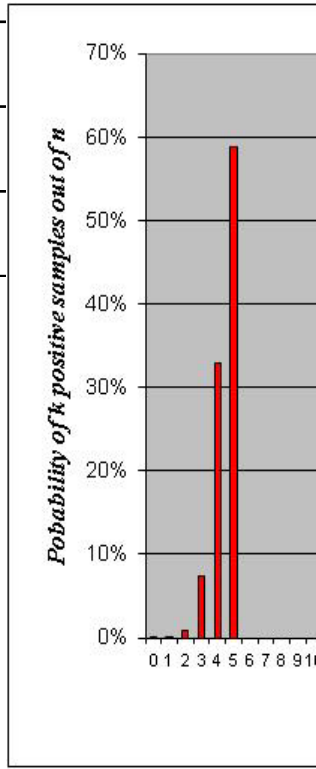
				No. of obtained positive seed subsamples							
				Laboratories							
	Seed Lot	**Contamination Level**	**No. of expected positive seed subsamples**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Alfalfa	Lot A	Healthy	0	0	0	0	0	0	0	0	0
Alfalfa	Lot B	Low	5	5	5	5	5	5	5	5	4
Alfalfa	Lot C	Medium	5	5	5	5	5	5	5	5	5
Faba bean	Lot D	Healthy	0	0	0	0	0	0	0	1	0
Faba bean	Lot E	Low	5	5	5	4	5	5	5	5	4
Faba bean	Lot F	Medium	5	5	5	5	5	5	4	5	5

Detection of Ditylenchus spp. by sieving method in faba bean seeds.



Detection of Ditylenchus spp. by sieving method in alfalfa seeds.





Validation test

Results PCR confirmation

Primers	Nematodes tested	No. of expected positive sample in PCR	No. of obtained positive seed subsamples Laboratories 1	2	3	4
Wood	*D. dipsaci*	10	5	10	10	5
Wood	*D. gigas*	10	9	10	10	7
Kerkoud	*D. dipsaci*	10	10	10	10	10
Kerkoud	*D. gigas*	10	10	10	10	9
Wood	Both	20	14	20	20	12
Kerkoud	Both	20	20	20	20	19

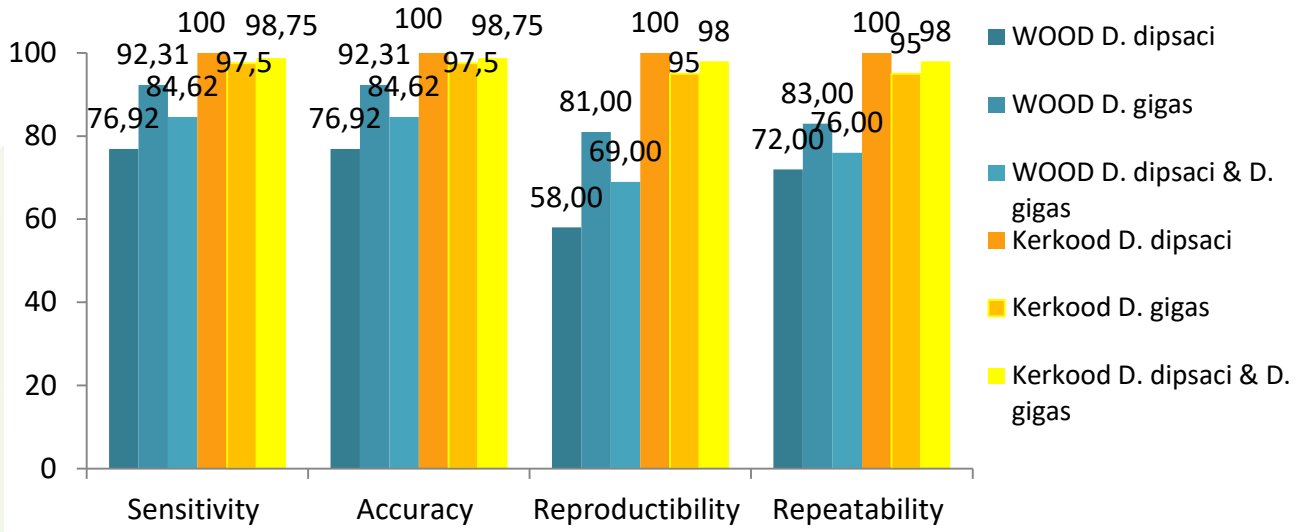
Kerkoud primers best results

97,5 % Accuracy

98,8% Sensitivity

98,0% Concordance

98,0 % Accordance



Summary

- Filtration method tested by 8 laboratories on 30 samples
- Results showed a very good sensitity, specificity, accuracy, concordance and accordance
  - On alfalfa seed lots (healthy, medium and high infected)
  - On faba bean seed lots (healthy, medium and high infected)

	Sensitivity	Specificity	Accuracy	Concordance	Accordance
Detection of *Ditylenchus* spp. by filtration method	>95%	>95%	>95%	>95%	>95%

- Primers from Kerkoud allow a better distinction between *D. dipsaci* and *D. gigas* only on individual nematode.

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Conclusion

- A detection method using filtration (sieve at 20µm) in order to concentrate population of nematodes present in a alfalfa and faba bean seed lot is validated.

- A PCR method using Kerkoud primers is validated in order to confirm the species of *Ditylenchus* between *D. dipsaci* and *D. gigas*

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Thanks for your attention