Seed testing preventing the introduction of quarantine pathogens

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Seed-borne plant pathogens can be easily moved around the world into new environments, which, if favourable to the pathogen, can lead to poor plant establishment as well as a reduction in the yield and quality of the crop (direct costs). Trade restrictions may be imposed on the affected country or region if the pathogen concerned is listed as a quarantine pest by other importing countries (reaction costs). Affected countries may have to implement stringent and costly control strategies to deal with outbreaks in order to regain lost export markets. As such, many countries require seed to be certified free from these pathogens as part of their phytosanitary import requirements. Several organisations and initiatives publish protocols to diagnose seed-borne pathogens, including the International Seed Testing Association (ISTA) and International Seed Health Initiative (ISHI). Other protocols are also published in handbooks (e.g. Albrechtsen, 2006; Mathur & Kongsdal, 2003; Saettler *et al.*, 1989) and scientific journals such as *Seed Science & Technology*. Unfortunately, many of these rely on destructively testing large numbers of seed, some of which, such as tomato and tree seed, can be extremely valuable. This can make some exports untenable on the grounds of cost and/or the availability of test seed.

Increasingly, many modern diagnostic technologies such as ELISA and real-time PCR are being used alongside traditional methods, including isolation and blotter tests, to aid pathogen diagnosis and reduce the spread of plant disease around the world. Non-destructive methods are also employed when available. Examples of EU quarantine seed-borne pathogens which are routinely tested for using modern diagnostic technologies include *Tilletia indica*, *Pantoea stewartii* and *Pepino mosaic virus*.

Tilletia indica causes the fungal disease of wheat and triticale known as Karnal bunt. Infected seeds may be partially bunted, containing many thousands of teliospores, or they may carry only a few teliospores on their surface. These teliospores have been shown to remain viable under European field conditions for at least 3 years. Further, it has been estimated that if *T. indica* occurs as a small (1000 ha) or large (50,000 ha) outbreak in the UK, it could cost the UK within the first year between 1.7 and 17.8 million Euro in direct, reaction and control costs (Sansford *et al.*, 2006). In 2004 the European Plant Protection Organization (EPPO) published its standard for diagnosis of *T. indica*, which has been adopted as the EU standard by many plant health laboratories. This involves sieving samples for teliospores, followed by their morphological and molecular assessment if required (Anon., 2004).

Pantoea stewartii, formerly known as *Erwinia stewartii*, causes bacterial wilt of maize, which is thought to be indigenous to America (Anon., 2006). It is thought that this pathogen is brought to new areas by seed and, once established, is spread by insect vectors (Anon., 1997). Various methods exist to diagnose infection in seed and frequently these are used in combination, as outlined in the EPPO standard for *P. stewartii* (Anon., 2006).

Pepino mosaic virus can infect a number of solanaceous hosts including pepino, tomato and potato. In tomato, spread to new areas is thought to be via infected seeds and seedlings and, once established in a crop, the virus is highly contagious and can lead to the downgrading of fruit, costing at least £16 m^{-2} based on 2005 prices (Spence *et al.*, 2006). Seed can be tested by a number of methods, including ELISA and real-time PCR. Traditionally this has been performed by destructive testing, but a recent industry-funded project has shown that non-destructive methods for diagnosis can also be employed (Mumford, 2006).

Ideally, as new methods are developed these should be made available to trade and government laboratories through peer-reviewed publications. Further, they should also be independently assessed through 'blind-testing' schemes to confirm their validity and to identify if they have advantages over established tests. However, the organisation and cost of running such schemes can be enormous, and the take-up of any new methods, particularly when involving technologies such as real-time PCR, may require high set-up and running costs. If these issues can be addressed, this will no doubt promote the uniformity and take-up of such methods and as a consequence reduce the spread and introduction of alien pathogens. The ability to test some seed lots using non-destructive methods, as highlighted by Mumford (2006), is also likely to bring many advantages to trade and governments alike.

References

Anon. (1997) EPPO datasheet on *Pantoea stewartii* subsp. *stewartii*. *Quarantine Pests for Europe*, CABI, UK

Anon (2004) Diagnostic protocol for regulated pests: *Tilletia indica*. *EPPO Bulletin*, **34**, 219–227.

Anon (2006) Diagnostic protocol for regulated pests: *Pantoea stewartii* subsp. *stewartii*. *EPPO Bulletin*, **36**, 111–115.

Albrechtsen, S E (2006) *Testing Methods for Seed-Transmitted Viruses: Principals and Protocols*. CABI Publishing, Wallingford, UK

Mathur S B; Kongsdal O (2003) *Common Laboratory Seed Health Testing Methods for Detecting Fungi*. International Seed Testing Association, Zurich, Switzerland.

Mumford R (2006) 'Wash and Grow'; the development of non-destructive tomato seed testing. HDC final report December 2006. Horticultural Development Council, East Malling, Kent, UK.

Saettler A W; Schaad N W; Roth D A (1989) *Detection of Bacteria in Seed and other Planting Material*. The American Phytopathological Society, St Paul, USA.

Sansford C; Baker R; Brennan J; Ewert F; Gioli B; Inman A; Kelly P; Kinsella A; Leth V; Magnus H; Miglietta F; Murray G; Peterson G; Porta-Puglia A; Porter J R; Rafoss T; Riccioni L; Thorne F; Valvassori M (2006) Pest Risk Analysis for *Tilletia indica* for the European Union. (2006). http://karnalpublic.pestrisk.net/

Spence N J; Basham J; Mumford R A; Hayman G; Edondson R; Jones D R (2006) Effect of Pepino mosaic virus on the yield and quality of glasshouse-grown tomatoes in the UK. *Plant Pathology*, **55**, 595–606.

A laboratory test to evaluate the selectivity of seed treatments in cereals

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Summary

Seed treatments are applied to a large proportion of certified seeds of cereals in France. Seed companies need information on the effectiveness and selectivity of seed-treatment products. Here, a laboratory method has been developed to evaluate the selectivity of seed treatments. This method can be used all year round, and is cheap compared with field trials. It requires the choice of susceptible seed lots and a germination test in suboptimal conditions (5°C for 4 weeks in sand at holding capacity). Selectivity is evaluated from the number of normal seeds in the test which result in dead seeds or in abnormal seedlings with short roots. This method is now used by seed-treatment producers in order to evaluate the selectivity of seed treatment formulations.

Introduction

Wheat (*Triticum aestivum*) is the most important seed production in France, with about 400,000 tons produced per year. Seed treatments are widely used, and many seed-treatment products are commercialised in France. Seed companies are interested in the effectiveness of seed treatments for protecting the seed and the emerging crop. But they also need to sell treated seeds with good vigour. Phytotoxic effects of seed treatments include poor germination, delayed establishment, and lack of first tiller. They may result from the selectivity of the seed treatment, susceptibility of the seed lot (about 5% seed lot tested), and suboptimal pedoclimatic conditions during germination and emergence. As field conditions favourable to phytotoxicity are difficult to anticipate, evaluation of selectivity in field trials is difficult to develop, and only a few results are workable. So to inform French seed companies about the selectivity of seed treatments commercialised in France, our laboratory developed, with the financial support of the Groupement National Interprofessionnel des Semences (GNIS), a specific method to evaluate the selectivity of seed treatments.

Materials and methods

Our method associates the choice of specific seed lots and a germination test in suboptimal conditions. Two seed lots, used in this study (L1 and L2), were chosen from several wheat seed lots harvested in different part of France in 2003. The standard germination of untreated seeds was above 95% (96 and 95.5%, respectively) and no fungal pathogen was detected by

sanitary analysis. Seed lot L1 was susceptible to seed treatment and seed lot L2 was only slightly susceptible.

Seeds were treated with four different treatments (TS1 to TS4) plus a reference seed treatment (Ref). The reference seed treatment presents a low selectivity. The registration of the four treatments was in progress when we initiated this study in 2004. The seed treatments were applied using a small-batch seed treater HEGE 11 by the slurry method at 1 l/q. After treatment, seeds were dried for 24 h at ambient temperature.

Two germination tests were conducted at LABOSEM with treated and untreated seeds. The standard germination test (7 days at 20°C in sand) according to International Seed testing Association (ISTA) recommendations was performed on 200 seeds. The other test is specific to evaluate the selectivity of seed treatment. This test is performed in sand at holding capacity at cold temperature (5°C) over 4 weeks. At the end of the 4 weeks, normal and abnormal seedlings and dead seeds were counted and typology of abnormal seedlings detailed. The test was performed on 200 seeds.

Statistical analysis was performed using the tolerance tables produced by ISTA. Those tables are based on the mean comparison of 200 seed results.

Results

In optimal conditions, germinations ranged from 90.5 to 97% (Figure 1). Seed lot L1 showed no significant difference of standard germination. The reference product applied on seed lot L2 induced a slight decrease in standard germination. The standard germination test cannot be used in order to evaluate selectivity of seed treatments.

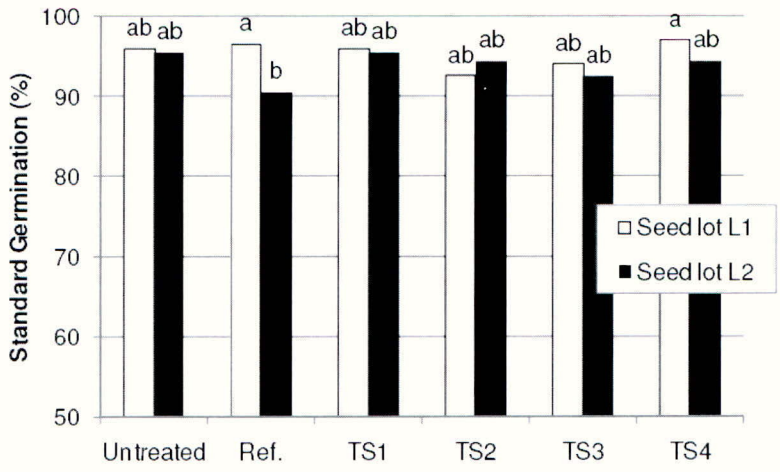

Figure 1 Standard germination (7 days at 20°C) of treated seeds of two seed lots. Different letters represent different germination results.

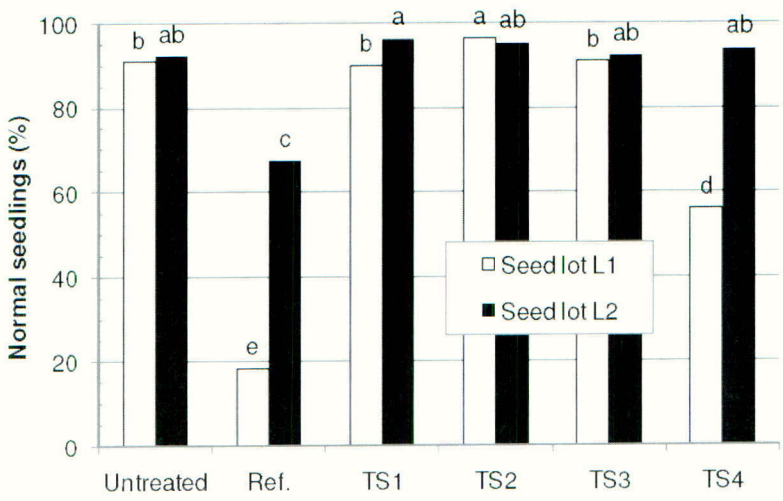

Figure 2 Normal seedlings after germination testing in suboptimal conditions (4 weeks at 5°C in sand at holding capacity). Different letters represent different germination results.

In suboptimal conditions, germination ranged from 18.5 to 96.5% (Figure 2). This test induced large differences in germination between seed lots and between seed-treatment products. Seed lot L1, treated with the reference or TS4, showed lower germination (18.5 and 56%, respectively) than ST1, ST2 and ST3. For the latter three treatments, germination was as good as for untreated seeds. Seed lot L2 showed a better overall germination. For this seed lot, only the seeds treated with the reference showed poor germination (67.5%).

The main cause of poor germination was the presence of abnormal seedlings with very short roots (less than 3 cm) compared with normal seedlings (about 6 cm long). This category of abnormal seedlings was the most frequent, and the treatments with the poorest germination were those with the highest percentage of abnormal seedlings with short roots.

Conclusion

A germination test in suboptimal conditions (cold temperature and sand at holding capacity) was developed at LABOSEM in order to evaluate the selectivity of seed-treatment products. Associated with a preliminary choice of seed lots, it gives unbiased results on the behaviour of new seed-treatment products. This test is more useful than field trials because discrimination of seed treatment selectivity is possible; the test can be used at any time during the year, and gives a result in a short time (4 weeks). This test was initially developed for the seed companies, but now is also used by seed-treatment producers in developing new formulations.

The effect of substrate when testing standard germination of treated maize seed

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Introduction

The rules of the International *Seed Testing* Association (ISTA, 2009) and of the American Association of Official Seed Analysts (AOSA, 1998) describe methods for standard germination (SG) testing of seed. Within the rules, testing conditions are specified for a wide range of species. For most species there are options with regard to the choice of substrate used during the test. These options have been validated internationally to show equivalent germination performance for seedlots under evaluation.

Over the past few years, reports of poor germination performance of maize seed in an SG test after the application of a systemic insecticide seed treatment were at odds with the successful field performance of those same seedlots. These reports came mainly from European countries in which sand is a popular substrate used in the SG test.

The objective of this small study was to compare the germination performance of a number of maize seedlots in an SG test after application with an insecticide using a number of different substrates.

Materials and methods

In two separate experiments, various maize (*Zea mays* L.) seedlots with germination above 90% were treated with CRUISER® (thiamethoxam, 0.63 mg/seed), then reassessed for germination using sand, rolled paper towels and compost (experiment 1 only). All substrates used are permitted for SG testing in the ISTA rules. Experiment 1 consisted of nine seedlots of the same variety and production year; experiment 2 consisted of 35 seedlots from the same production year, but a range of varieties. The seedlots were grouped according to vigour (high, medium and low) based on germination performance of untreated seed in a rolled-towel cold test (Hampton & Tekrony, 1995).

Results and discussion

In experiment 1, it made no difference to the final germination of untreated seed which substrate was used (Figure 1). However, when the same seedlots were tested after treatment with thiamethoxam, the substrate used clearly influenced final germination – when tested in sand, mean germination values were lower than when tested in either compost or in rolled-paper towels. Since only one variety was involved, the experiment was repeated with a group of seedlots that ranged in vigour level and represented a number of different varieties.

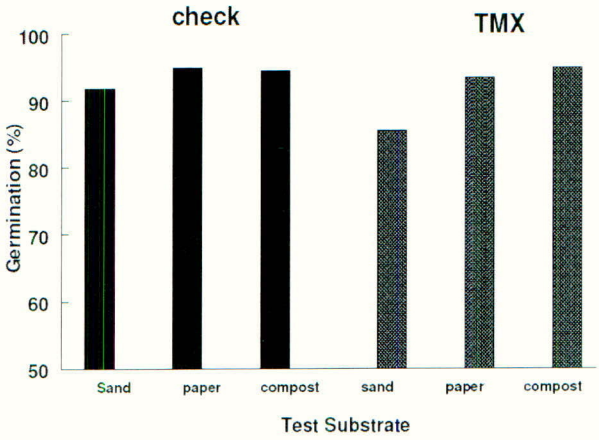

Figure 1 Germination performance of nine seedlots of the same variety before (check) and after application of a thiomethoxam (TMX) seed treatment in the standard germination test, using sand, paper (rolled towels) and compost as substrates.

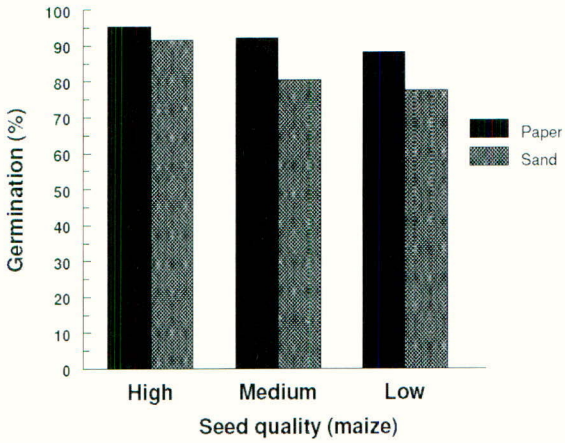

Figure 2 Germination performance of 35 maize seedlots of various varieties, treated with thiomethoxam (TMX) in the standard germination test using sand and paper (rolled towels) as substrates (n = 2 for high-vigour seedlots, 27 for medium- and 7 for low-vigour seedlots).

When high-vigour seed was tested (experiment 2), it made no significant difference to germination performance whether sand or paper was used as substrate (Figure 2). As seed vigour decreased, however, the impact of substrate on germination performance was noticeable. Mean germination of rolled-paper towel tests was 11.5% higher than sand (80.5 versus 92.0%, respectively).

This represents the difference between a saleable seedlot and one that is not, thus the choice of substrate when subjecting treated seed to the SG test is clearly important, as it has the potential to substantially influence results.

References

AOSA (1998) *Rules for Testing Seeds*. American Association of Official Seed Analysts, Lincoln, NE, USA.

Hampton, JG; TeKrony DM (1995) *Handbook of Vigor Test Methods*, 3rd edn. International Seed Testing Association, Zurich, Switzerland.

ISTA (2009) Chapter 5: The germination test. In *International Rules for Seed Testing*. International Seed Testing Association, Bassersdorf, Switzerland.

An electrotherapy technique for eliminating a major seed-borne virus of common bean

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Summary

Bean common mosaic virus (BCMV) is a major seed-transmitted virus of dry and snap beans. The use of virus-free germplasm is a prerequisite for production of certified seeds, which is an important disease control measure. In this study, the efficiency of electrotherapy was evaluated for eliminating BCMV and producing virus-free plants in common bean. Nodal cuttings of infected bean plants were exposed to electric currents of 5, 10 and 15 mA for 10 min and planted *in vitro*. One-month-old regenerated plantlets were tested using a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) method for BCMV infection. The electric current of 15 mA for 10 min produced the highest percentage of BCMV-free bean plants. The technique developed in this study may be a useful procedure for production of virus-free germplasm and/or nucleus seed stock in crop plants infected with seed-borne viruses.

Introduction

Bean common mosaic virus is the main seed-borne virus of common bean, and can be easily transmitted through the generations at a very high rate (Morales & Bos, 1988; Hormozi-Nejad *et al.*, 2008). Due to its high seed transmissibility, BCMV is widely spread throughout the world and results in considerable yield losses (Drijfhout, 1978; Mavric & Vozlic, 2004). Seed transmission of BCMV varies in different genotypes, ranging from 0 to 83% (Morales & Bos, 1988; Hormozi-Nejad *et al.*, 2008). Therefore development of virus-free germplasm is considered one of the main practical approaches for the control and management of BCMV disease and production of a high-quality common bean crop. Various methods have been employed to eliminate viruses from plants, including meristem culture and thermotherapy. These methods are technically demanding and time-consuming. Electrotherapy, however, is a simple method of virus eradication without the need to use any special or expensive equipment. In this technique, an electric current is applied to plant tissues in order to disturb viral nucleoprotein and its virulence activity. The method was first employed for elimination of PVX from potato plants in which an electric current of 15 mA for 5 min led to 60–100% PVX elimination in various cultivars (Lozoya *et al.*, 1996). Electrotherapy has also been used successfully for elimination of PVX, PVY, PVA, PVS and PLRV (Pazhouhandeh & Mozafari, 2001). The most effective treatment in this study was also 15 mA for 10 min with 54–85% plant regeneration and 26–100% virus elimination rates. Here we report application of the electrotherapy technique for elimination of BCMV from bean tissues for the first time.

Materials and methods

Ten-day-old greenhouse-grown plants of two bean cultivars, Khomein and Capsouli, inoculated by BCMV isolate were collected from the main bean-growing regions in Central Iran as previously described (Kaiser *et al.*, 1968). Plants were assayed by DAS-ELISA for the presence and concentration of BCMV 21 days post-inoculation. Stem segments of infected plants were used for electrotherapy. Stem segments containing three or four axillary buds were immersed in TAE buffer in an electrophoresis tank and exposed to electric currents of 5, 10 and 15 mA for 10 min using a Biometra power supply. Immediately after treatment, the stems were surface-sterilized using 70% ethanol for 30 s, followed by 0.5% sodium hypochlorite for 3 min. Stem segments were subsequently rinsed with distilled water three times. Explants were prepared by dividing stem segments into nodal cuttings with a single axillary bud. Explants were cultured in test tubes containing MS5 medium (Benedicic *et al.*, 1997) and maintained in a growth chamber under light conditions of 16 h light and 8 h darkness at 23–25°C. Control explants with no electrotherapy were also planted on the culture medium, as explained above. After 30 days, the plant regeneration rate was estimated by counting the regenerated plantlets versus the total cultured explants in each treatment. Regenerated plantlets were assayed for BCMV infection by a DAS-ELISA technique (Clark & Adams, 1977). The BCMV-negative samples were considered as virus-free plantlets. The effect of a treatment on production of virus-free plantlets is influenced by both plant regeneration rate and the rate of virus elimination. A therapy efficiency index (TEI) was estimated for each electrotherapy treatment:

$$\text{TEI} = \text{percentage of regenerated plantlets} \times \text{percentage of virus-free samples.}$$

Results and discussion

Application of the electrotherapy technique on the two bean cultivars Khomein and Capsouli resulted in successful elimination of BCMV from bean plants. ANOVA of virus elimination rates revealed a significant difference between the three electrotherapy treatments (5, 10, 15 mA) for 10 min. ANOVA also showed that the two bean cultivars were not significantly different in responding to electrotherapy. The efficiency of electrotherapy in producing virus-free plants depends on both plant regeneration and virus elimination rates.

Plant regeneration

Electric currents may adversely affect the survival of explants and, as a result, plant regeneration. There was an obvious reduction in plant regeneration on increasing the intensity of the electric current. A plant regeneration rate of 58.4 to 79.5% was observed in the two cultivars used in this study. Three electric currents of 5, 10 and 15 mA resulted in 79.5, 68.6 and 58.4% mean plant regeneration, respectively (Table 1). Regenerated plantlets from electrotherapy were morphologically similar to plants regenerated from non-treated control explants.

Virus elimination and therapy efficiency

The mean virus elimination rates of the two cultivars for three electric currents of 5, 10 and 15 mA were 37.2, 45.5 and 71.9%, respectively (Figure 1). The highest virus elimination rate was seen when explants were exposed to 15 mA. The number of ELISA-negative samples was increased when the electric currents were raised. This implies that there should be an optimum electric current in which the highest rate of virus elimination and plant regeneration is obtained for each cultivar. Therefore both of these factors have been taken into consideration, and a

Table 1 Effects of electrotherapy treatments on *in vitro* plantlet regeneration and BCMV elimination rates of two bean cultivars

Cultivar	Treatment (mA/min)	Regeneration		Elimination		Therapy efficiency
		Number[1]	%	Number[2]	%	
Khomein	0/0	17/20	85	–	–	–
	5/10	38/47	80.8	11/38	40	28.9
	10 /10	27/41	65.8	12/27	44.4	29.2
	15/10	26/49	53.6	19/26	73	39.1
Capsouli	0/0	18/20	90	–	–	–
	5/10	29/37	78.3	10/29	34.4	26.9
	10 /10	30/42	71.4	14/30	46.6	33.3
	15/10	31/49	63.2	22/31	70.9	44.8

[1]Regenerated plantlets/number of treated explants.
[2]BCMV-free plants/number of regenerated plantlets.

therapy efficiency index (Lozoya *et al.*, 1996) has been defined for each treatment. The TEI for the three electrotherapy treatments of 5, 10 and 15 mA was estimated as 28.9, 29.2 and 39.1 in cv. Khomein, and 26.9, 33.3 and 44.8 in cv. Capsouli. The electric current of 15 mA for 10 min resulted in the highest TEI in both cvs Khomein and Capsouli.

Previous reports suggested that an improvement of plant regeneration might be happening while explants were exposed to a mild electric currents, prior to *in vitro* culture (Goldsworthy, 1987; Lozoya *et al.*, 1996). However, based on the results obtained in this study, higher levels of electric current reduce plant regeneration. This study also clearly indicated that, although

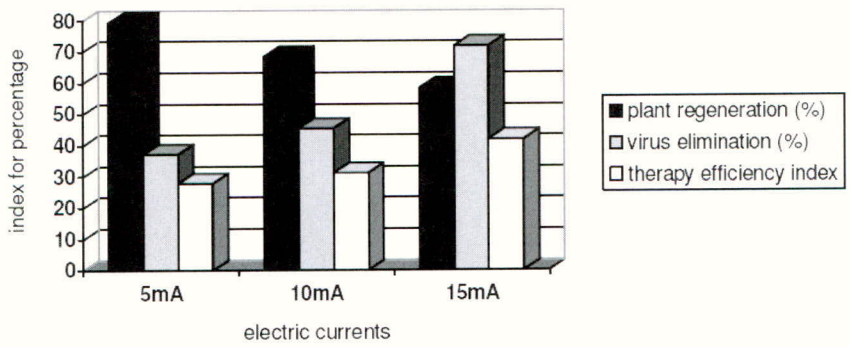

Figure 1 Values of plant regeneration, virus elimination and therapy efficiency index of three electrotherapy treatments

the regeneration rate declined with increasing electric currents, the TEI of each treatment shows the actual value for treatment efficiency.

This study has revealed that electrotherapy is an effective technique for elimination of BCMV from bean plant tissues. Although electrotherapy is not always more effective than other virus elimination methods, this technique appears to be comparatively simple, fast, and easier than other techniques. With these characteristics, electrotherapy may also be used to eliminate of other seed-borne viruses from plants.

References

Benedicic D; Ravnikar M; Gogala N (1997) The regeneration of bean plants from meristem culture. *Phyton: Annales Rei Botanicae*, **37**, 151–160.

Clark MR; Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, **34**, 475–483.

Goldsworthy A (1987) Electrical stimulation of tissue culture growth and morphogenesis. *Agricell Report*, **8**, 14.

Hernández PR; Fontanella J; Noa JC; Pichardo T; Igarza Y; Cardenas H; Manso R (1997) Electrotherapy: a novel method for eliminating viruses from garlic. *Horticultura Argentina*, **16**, 68–71.

Hormozi-Nejad MH; Mozafari J; Rakhshandehroo F (2008) Production and certification of virus-free nucleus seeds in common bean. *Proceedings of the First National Congress on Seed Science and Technology, 12–13 November 2008*. Gorgan, Iran.

The development of an ipconazole microemulsion formulation for seed treatment

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Summary

The benefits of microemulsion (ME) technology to seed treatment formulations are demonstrated through the physical performance characteristics of Ipconazole 15 ME (Rancona™ 15 ME), a new low-active seed treatment fungicide. The advantages of this formulation type are illustrated by comparison with a typical commercial flowable (FS) seed treatment product. Differences in the inherent rheological properties of the two types of formulation have been determined. Product storage stability, pourability, rinsibility and seed treatment efficiency are compared between the ME and FS formulations, and advantages shown by the ME are related to the rheological properties

Introduction

The convenience of liquid formulations for seed treatment is the main reason why the leading commercial formulations today are based on the suspension of solid particles and/or emulsion droplets in water; formulation types include flowables (FS – solid active ingredient particle suspension), emulsions (ES – emulsion droplet suspension with dissolved or neat active ingredient) and suspoemulsions (SE – active ingredient in solid and droplet suspension) (Anon., 2002). The common feature of all these formulation types is that due to a density difference between the particle/droplet and water, there is a tendency for the active ingredient to sediment or cream on storage, which requires rehomogenisation before the product can be used. Advances in suspension technology, combined with improvements in milling and emulsification techniques, have enabled the development of products that show reasonable stability over 2–3 years and at typical storage temperatures. Suspension stability is achieved by the incorporation of viscosity modifiers into the formulation; these are based on clays or natural polymers that self-associate in water. In order to obtain a good seed treatment product, the level of viscosity modifier must be carefully controlled, sufficient to give a structure capable of suspending particles, but not so much that the product is too viscous resulting in poor pumping and atomisation characteristics. In practice, few current commercial FS products are perfect in either respect and will show some settling on storage, as well as being more viscous than required for ideal pumping characteristics. A particular problem encountered when using flowables for seed treatment is the requirement for pump recalibration when ambient temperatures change between applications, altering the pumping characteristics of the product.

Ipconazole (Figure 1) has recently been registered for use as a low-dosage seed treatment fungicide for wheat and barley. It has a broad fungal spectrum and displays excellent crop and operator safety characteristics.

As it is effective at very low application rates, it has been possible to incorporate ipconazole into a novel microemulsion (ME) formulation: Ipconazole 15 ME contains 15 g/l of ipconazole and is typically applied at between 1 and 1.3 litres/tonne of seed, much less than for many other azoles. The biological performance of this product is reported elsewhere in this Proceedings (Tomkins, 2009). This paper explores the advantages of formulating ipconazole as an ME, comparing it with a conventional FS.

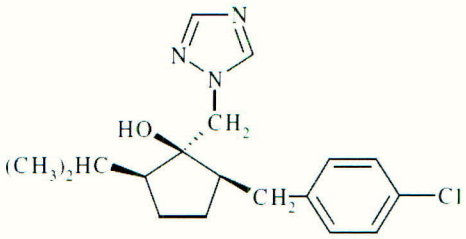

Figure 1 Ipconazole: systemic and contact fungicide

An ME is defined as 'a clear to opalescent oil and water containing liquid, to be applied directly or after dilution in water'(Anon., 2002). Microemulsions comprise very small surfactant/oil droplets (typically 0.01–0.1 µm) which are much smaller than conventional emulsion droplets (0.1–10 µm) and in which the active component is solubilised. ME formulations are transparent due to the small droplet size (Figure 2), in contrast to flowables and emulsions, which are opaque due to the larger particle/droplet size. Being thermodynamically stable, they show no tendency to phase-separate (droplet coalescence/creaming) (Knowles, 2005). For this reason, MEs do not require viscosity modifiers to keep the droplets in suspension and they can be formulated to be 'water thin'.

In addition to the active ingredient, Ipconazole 15 ME comprises a surfactant component, an organic solvent, an antifoam, an antifreeze agent, water, and a food-approved, water-soluble red dye. The formulation has been optimised by experimental design to ensure complete solubility of the ipconazole in the surfactant/solvent microemulsion phase together with complete solubility of the dye in the aqueous region of the microemulsion. In this study, Ipconazole 15 ME is compared with a commercial flowable formulation (FS1), which was chosen as typical in terms of the physical properties of this formulation type. It comprises the active component as solid particles suspended in an aqueous phase that has been thickened by viscosity modifiers to keep the particles in suspension. The red coloration in the formulation is provided by suspended solid pigment particles.

Methodology

Stability studies

The stability of Ipconazole 15 ME was confirmed by storage stability studies. Approximately 25 g of formulation was stored in 30 ml glass winchesters in temperature-controlled incubators

FS: solid active ingredient particles (0.1–10 µm) dispersed in water

ME: active ingredient solubilised in surfactant/ solvent droplets (0.01–0.1 µm) in water

Figure 2 Comparison of FS with ME

at –10, 0, 25 and 50°C for between 2 weeks and 24 months. Another sample was subjected to five freeze/thaw cycles (–30°C/ambient) then stored for 4 weeks at 40°C. At defined periods, the samples were tested for stability by a number of tests, including the following.

- A visual observation to look for signs of precipitation or crystallisation; separation into more than one phase; colour change. The sample was then inverted gently 10 times to ensure homogenisation and the following tests performed.
- Measurement of the ipconazole assay by high-performance liquid chromatography (HPLC) or the active in FS1.
- Measurement of the pH of a 1% solution in distilled water.
- The dilution stability of the product. This was achieved by injecting 2 ml product into a 100 ml glass centrifuge tube containing 98 ml WHO (World Health Organization) standard water at 342 ppm hardness. The quality of initial dispersion was judged by eye as the formulation mixed with the water; the centrifuge tube was then inverted 10 times to complete the dispersion (if necessary), after which it was left to stand over a 24-h period. The stability of the diluted sample was determined by examining the centrifuge tube at regular intervals, looking for any phase separation within the liquid, including the separation into more than one liquid layer or the sedimentation of precipitated solid particles or crystals. Phase separation was quantified using the graduations of the centrifuge tube.

Rheology studies

The rheological properties of Ipconazole 15 ME and FS1 were compared using a Haake Rheostress 600 rheometer controlled by Haake RheoWin3 Job Manager software. Measurements were made by placing the liquid between a 6 cm cone and plate configuration with a 0.105 mm gap, and increasing the shear rate from 0.001 to 550 s^{-1} in logarithmic increments over a period of 3 min at 20°C. The shear stress was measured and the results plotted as the change in viscosity with shear rate. (Note: viscosity is calculated from the relationship viscosity = shear stress/shear rate.) The work was then repeated at 50°C with the same protocol.

Finally, the change in viscosity of Ipconazole 15 ME with temperature was determined over the temperature range 20 to –10°C, and again a comparison was made with FS1. Measurements were made using a 3.5 cm cone with a 0.105 mm gap. The instrument was run at a constant shear rate of 10 s^{-1} for 20 min as the temperature was decreased at a steady rate from 20 to –10°C. The shear stress was measured and the results plotted as the change in viscosity with temperature.

Pourability and rinse residue

The pourability and rinsibility at 20 and 0°C of Ipconazole 15 ME and FS1 from 1-litre HDPE containers were determined following CIPAC MT 148; the product was poured from a full container inverted at an angle of 45° for 60 s, followed by complete inversion for a further minute. The percentage weight of product remaining in the container was then weighed and calculated to give the pourability. The rinsibility was measured by addition of distilled water to the container, followed by 10 inversions and pouring out the rinse water by the same method. The percentage weight of retained product gave the rinsibility.

Seed treatment

A winter wheat cultivar (Savannah) was treated with 1.3 ml/kg Ipconazole 15 ME; 7.8 ml of a 1:2 mixture with water was added to 2 kg wheat in a Rotogard R300 laboratory seed treater using a Volpec multipipette. Addition was over a period of about 10 s, with the rotor speed set at 45 Hz. For comparison, the same seed treatment process was repeated with 2.5 ml/kg FS1; 10 ml of a 1:1 mixture with water was added to 2 kg wheat. Both applications were typical of commercial rates.

In order to measure the uniformity of the treatment over the seeds, a single seed-loading experiment was carried out. Forty-four single seeds were randomly selected from the treated sample and weighed into individual vials. Each single seed was extracted by acetone and the extract analysed for ipconazole by gas chromatography. The weight of ipconazole on each seed was then determined as a fraction of the target weight expected per seed based on the dosage used in the treatment. Likewise, 44 seeds treated with FS1 were analysed for the active fungicide in this product and the fraction of the target weight calculated.

Results

Storage stability

The results of the storage stability for Ipconazole 15 ME and FS1 are given in Tables 1 and 2, respectively. The microemulsion formulation showed excellent chemical and physical storage stability up to 24 months and over a wide range of temperature conditions. There was no phase separation, precipitation or crystallisation in any of the stored samples, nor in the product when it was diluted in water. The ipconazole assay and pH remained constant within the limits of experimental error. Whilst the FS formulation showed acceptable chemical stability, it also demonstrated some physical instability that is typical of this class of formulation. Over a period of time, a clear bleed layer appeared at the top of the product, as particles of active ingredient and pigment settled under gravity. Settling of particles was also evident when the formulation was diluted in water. The fact that the dilution separation did not increase with product storage

Table 1 Storage stability of Ipconazole 15 ME

Parameter	Initial	Freeze/ thaw	2 mths, 50°C	3 mths, 0°C	3 mths, –10°C	6 mths, 25°C	24 mths, 25°C
Appearance	DRT	DRT	DRT	DRT	DRT	DRT	DRT
Bleed	None	None	None	None	None	None	None
Sediment	None	None	None	None	None	None	None
Dilution separation 2 h (ml)	0	0	0	0	0	0	0
Dilution separation 24 h (ml)	0	0	0	0	0	0	0
% ipconazole (w/w)	1.40	1.40	1.40	1.40	1.40	1.43	1.42
pH (1% soln)	6.5	6.8	6.7	7.1	6.9	6.9	6.5

DRT, dark red translucent.

Table 2 Storage stability of commercial flowable FS1

Parameter	Initial	Freeze/ thaw	2 mths, 50°C	3 mths, 0°C	3 mths, –10°C	6 mths, 25°C	24 mths, 25°C
Appearance	MO	MO	MO	MO	MO	MO	MO
Bleed	None	8%	5.8%	Trace	4.3%	3.8%	26.5%
Sediment	None	None	None	None	None	None	None
Dilution separation 2 h (ml)	0.5	0.6	0.8	0.5	0.5	0.7	0.6
Dilution separation 24 h (ml)	1.0	0.9	0.9	0.9	0.9	0.9	0.8
% active (w/w)	35.3	35.3	32.2	35.2	35.3	33.1	34.0
pH (1% soln)	7.9	7.1	7.4	7.9	7.9	7.5	7.4

MO, magenta opaque.

time or different storage temperatures implied that the particles were well dispersed in the formulation and that the settling which produced the bleed was due to insufficient or contracting structure provided by the viscosity modifiers. This effect is known as syneresis. (Note: in a separate study, it was found that the particle size distribution in FS1 and its homogenised viscosity remained constant over 24-month storage and at different temperatures, which supports the model of good dispersion but imperfect suspending structure.)

Rheology

Figure 3 shows the change in viscosity with shear rate of Ipconazole 15 ME at 20°C and compares it with FS1. At all shear rates, the microemulsion has significantly lower viscosity than the flowable. The ipconazole microemulsion behaves like water, demonstrating constant viscosity at all shear rates (Newtonian behaviour). This is to be expected as it is a clear solution with very small droplets. In contrast, the flowable formulation shows very high viscosity at low shear rates with much lower viscosity at high shear rates. This rheological behaviour is deliberately tailored for flowables by inclusion of viscosity modifiers such as clays and biopolymers in the formulation. The 'structure' provided by the viscosity modifiers gives

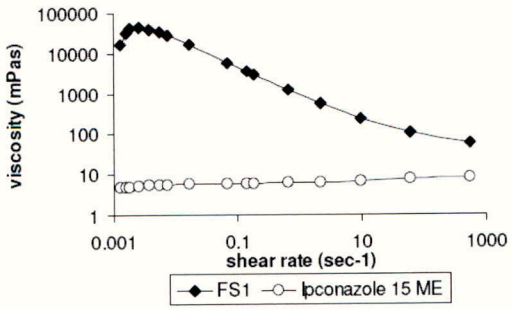

Figure 3 Change of viscosity with shear rate at 20°C

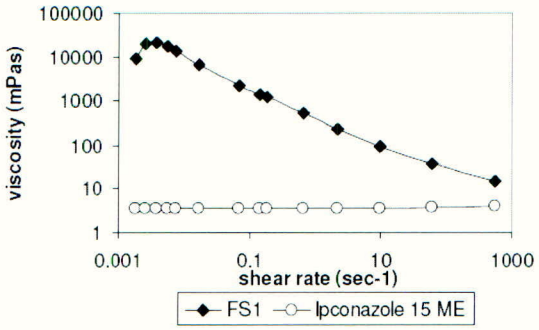

Figure 4 Change in viscosity with shear rate at 50°C

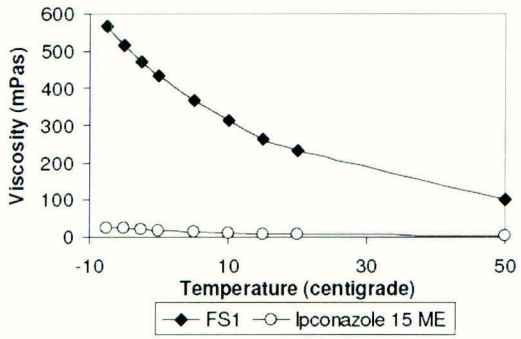

Figure 5 Change in viscosity with temperature at 10 s^{-1}

a sufficiently high viscosity at low shear rate to enable small particles of active ingredient and pigment to be suspended in the aqueous phase; the gravitational force on the particles is insufficient to break the structure, so that rapid settling is prevented. The formulations can be described as shear thinning because the structure breaks at high shear rates and viscosity is considerably reduced; this enables the formulation to be easily pourable. Whilst viscosity modifiers significantly reduce the rate of settling in products containing suspended particles, there is a tendency for their structure to rearrange over a period of time, with the structure contracting. This causes the bleed (syneresis) shown by FS1 (Table 2), as the suspended particles are dragged down with the structuring agents. The rate of bleed can be reduced by increasing the concentration of structuring agents, but this has the adverse effect of increasing the pouring viscosity (Hughes, 2005).

Figure 4 compares the rheology of the two products at 50°C. Whilst the viscosity of the ipconazole microemulsion differs little from that at 20°C, the viscosity of the flowable at 50°C is reduced to approximately half its value at 20°C for a given shear rate. This reduction in viscosity partly explains why the rate of bleed observed in FS1 is faster at higher temperatures; the structure is weaker and its reorganisation is more rapid.

Figure 5 compares the change in viscosity with temperature at a fixed shear rate (10 s^{-1}) for the two systems over the range –7 to 50°C. The changes observed for the ipconazole microemulsion are minimal and it is 'water thin' over the complete range. In contrast, the flowable formulation shows considerable variation in viscosity with temperature.

The effect of these differences in rheological behaviour between the microemulsion and flowable on important aspects of seed treatment are explored below.

Table 3 The pourability and rinsibility of Ipconazole 15 ME and FS1

Product	Temperature (°C)	Pourability (% retained)	Rinsibility (% retained)
Ipconazole 15 ME	16.9	0.25	0.09
Ipconazole 15 ME	1.5	0.23	0.10
FS1	16.9	1.12	0.12
FS1	1.5	1.36	0.13

Pourability and rinsibility

The results (Table 3) show that the higher viscosity of FS1 gave a significantly higher retained residue in the 1 litre container than the microemulsion, which can be explained by the larger low shear viscosity causing clinging to the container wall. Whilst the level of retained Ipconazole 15 ME did not change with temperature, an increase in residue was observed for FS1 at low temperature. The percentage weight of product retained after rinsing was low for both products, indicating that both disperse well.

Seed treatment

Figures 6 and 7 show that Ipconazole 15 ME gave a much more even coverage of the seed surface with active product than was observed for FS1. 90% of the seed were within 80–120% of the target coverage with the microemulsion formulation. For the flowable, only 60% of the seed were within this range. This even coverage is particularly impressive given the low applied dosage for Ipconazole 15 ME (7.8 ml, cf. 10 ml for FS1), and is probably explained by the low viscosity of the microemulsion giving a finer droplet size from the spinning disk atomiser in the treater and also providing rapid spreading and redistribution when the droplets impact with the seed surface. The higher surfactant level in microemulsion formulations is also likely to assist spreading on the seed surface through reduced dynamic surface tension.

Discussion

This work has shown that microemulsions can be formulated at a significantly lower viscosity than flowables, and that this viscosity remains virtually constant for an ME over a broad range of temperatures and applied shear rates. In addition, Ipconazole 15 ME has been shown to be

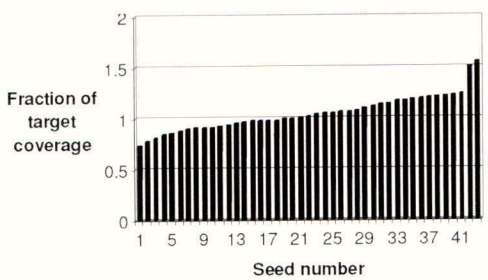

Figure 6 Seed treatment of winter wheat with Ipconazole 15ME

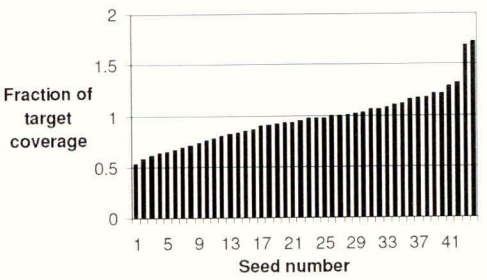

Figure 7 Seed treatment of winter wheat with FS1

more physically stable than a typical FS formulation, which can be explained by nanoscale dispersion of the active ingredient in its microemulsion droplets. This was reflected in the microemulsion showing no separation on storage or dilution, in contrast to the flowable, which showed significant bleed after long-term storage and under freeze/thaw conditions in particular. Significantly better pourability and seed coverage has been demonstrated for Ipconazole 15 ME, and this has been explained in terms of the measured differences in rheology between the ME and FS. These results provide a good explanation for the following observed advantages for microemulsion technology in seed treatment.

- When treating seed with flowables, there is frequently a requirement to recalibrate the pump delivering product to the point of application. This can be explained by changes in the ambient storage temperature of the product causing a change in the liquid viscosity from one day to the next. Assuming laminar flow, the pressure drop in circular pipes can be expressed as $\Delta p = 32\eta L\rho\ \omega/D^2$ where p = pressure drop; η = kinematic viscosity; L = pipe length; ρ = density; ω = flow velocity; D = pipe diameter. Hence, if the pump delivers a particular pressure to a liquid in a fixed length of pipe, the flow velocity will drop if the viscosity increases; this will typically happen to a flowable if its temperature drops. Recalibration of a pump when using a flowable may also be more difficult due to the variation in viscosity with shear rate. At very low temperatures, it may become impossible to pump a flowable due to excessive viscosity or there may be a danger of pipe splitting due to excessive pressures.
- Little recalibration is expected for a microemulsion formulation because its viscosity remains approximately constant with changing temperature. It will also be easily pumpable at all temperatures above its freezing point (–10°C for Ipconazole 15 ME). The low viscosity and easy flow properties of the microemulsion should reduce any problem of spray nozzle blockage encountered in the seed treater.
- The fact that an even coverage of the seed was obtained with a low volume of applied liquid for the microemulsion enables the low application rate for Ipconazole 15 ME to give efficient and accurate seed protection without the need for large dilution with water in the seed treater.
- The low viscosity of Ipconazole 15 ME ensures very low residue in containers, reducing product wastage and the quantities of rinse residue requiring disposal, particularly in low-temperature conditions. Cleandown is further simplified for the microemulsion as the formulation when dried onto seed treater surfaces is easily rinsed with a minimum of water. Efficient run-off from surfaces gives a clear visual indication of microemulsion product quantities remaining in transparent containers.
- The excellent storage stability of the microemulsion avoids the need for rehomogenisation, which can be a difficult process for flowables in large containers such as intermediate bulk carriers.

Conclusion

The use of a microemulsion seed treatment product offers high-quality seed coverage due to accurate calibration and delivery. There are environmental benefits as a result of reduced waste from equipment cleandown and from container rinsing. All the factors highlighted above should also reduce worker exposure to chemicals during seed treatment. The low required

application rate for ipconazole enables it to be formulated into a product that is easy to transport and handle and that has reduced environmental impact. These benefits are maximised through the selection of an ME formulation.

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References

Anon. (2002) *Catalogue of Pesticide Formulation Types and International Coding System*, Technical Monograph No. 2, 5th edn. CropLife International, Brussels.

Hughes R (2005) Practical rheology. In Cosgrove T (ed.) *Colloid Science: Principles, Methods and Applications*. Blackwell, Oxford, 201–227.

Knowles A (2005) New developments in crop protection product formulation. *Agrow Reports*, T&F Informa, UK.

Tomkins M (2009) The development of seed treatment products based on the new fungicide ipconazole. In Biddle AJ (ed.) *Seed Production and Treatment in a Changing Environment*, Symposium Proceedings No. 83. British Crop Production Council, Alton, UK.