Phomopsins: an overview of phytopathological and chemical aspects, toxicity, analysis and occurrence.

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Abstract
Phomopsis leptostromiformis, and its teleomorph *Diaporthe toxica,* is a lupin pathogen that causes stem blight in young lupins and, as a saprophyte, has been detected on dead lupine material. Under favourable conditions, the fungus produces phomopsins (PHOs), a family of macrocyclic hexapeptide mycotoxins capable of binding tubulin through the tripeptide side chain. The toxic effects appear largely confined to the liver. In particular, the ingestion of PHO contaminated lupin stubble has been linked to lupinosis, a debilitating disease of sheep (the most sensitive animal) characterised by disorientation, blindness, lethargy, and eventually death. The chemical structure of PHO A, B and D has been identified. Analytical methods to determine PHOs are mainly enzyme-linked immunosorbent assays or chromatographic separations, in combination with ultraviolet and mass spectrometric detection. The data about the PHOs occurrence are limited to Australia, restricted to lupin seed. Only one survey has been carried out on lupin seeds and flours from Swiss market. Not many strategies have been developed to limit contamination of lupin seed. Efforts devoted to control lupinosis in Australia focused on the development of cultivars resistant to *Phomopsis* infection. There are few examples in literature of decontamination or detoxification of PHOs; moreover, they have been shown to be resistant to extensive processing, including cooking. Australia and New Zealand are the only countries that included PHOs in their mycotoxin regulations, with a limit of 5 μg/kg in lupin seeds and derived products.
Phomopsins are poorly studied mycotoxins and risk assessment on PHOs has not been done at the European level. The collection of all available scientific data was requested by EFSA in a specific project and partners involved considered it of general interest preparing this review to highlight the limited available information, which indicate that the assessment of potential risk related to PHOs is currently not feasible.

Keywords: *Phomopsis leptostromiformis, Diaporthe toxica*, PHO, lupin, lupinosis.

Introduction

Phomopsins (PHOs) are among the poorly studied mycotoxins. Risk assessments on PHOs have not been done at the European level and therefore it is expected that the European Commission may ask EFSA to assess the risks to human and animal health related to these substances in the near future. To carry out these risk assessments to the highest standards and in an efficient way, scientific background information are needed. Consequently, in 2008, the European Food Safety Authority (EFSA) funded a project for the collection of scientific data

on several mycotoxins (included PHOs) and other natural plant toxicants. Partners involved in this project considered interesting to prepare this review to state the available knowledge; it focuses on PHO production by *P. leptostromiformis*, structure, biosynthesis, analysis, natural occurrence in food and feed, mitigation, toxicity and pharmacokinetic.

Phomopsis leptostromiformis

Phomopsis (*Diaporthe toxica* Punith, anamorph *Phomopsis leptostromiformis* (Kühn) Bubák) is a pathogen of lupin, a grain legume crop grown in numerous Mediterranean-type climatic regions. From the genus *Lupinus* more than 400 species are known, of which only four are of agronomic interest: *L. albus, L. angustifolius, L. luteus* and *L. mutabili*. They are used as green forage, as manure, or are intended for human nutrition (seed). In Mediterranean countries and in South America, lupins are a very common snack. They are normally soaked in sea water for 2-3 hours, to mellow the flavour, and consumed raw.

In 2009, 934,225 tons of lupins have been produced worldwide. The main producing country is Australia (*L. angustifolius,* 614,000 tonnes), followed by several Central and Eastern European countries including Belarus (73,708 t), Poland (57,000 t), Germany (50,000 t) and Ukraine (26,800 t) cultivating *L. luteus* and *L. albus.* Another important producer country is Chile with approximately 31,623 t produced in 2009, specialized in the cultivation of *L. albus* (FAO, 2010).

Phomopsis leptostromiformis was first detected in Poland on yellow lupine plants in the early twentieth century (Kochman, 1957). This organism has been reported to cause stem blight in young lupins (*Lupin luteus*) (Ostazeski and Wells, 1960): it produces sunken, linear stem lesions that contain black, stromatic masses, and it also affects the pods and seeds. The fungus is also a saprophyte and grows well on dead lupine material (e.g. haulm, pods, stubble) under favourable conditions (Merck, 2008). It produces phomopsins as secondary metabolites on infected lupine material, especially after rain (Culvenor *et al.*, 1977).

Regarding other potential PHOs producer fungi, only two references are available: *P. emicis* has been reported to produce PHO A on *Emex australis* (Shivas *et al*., 1994), while *P. castanea* has been considered a possible PHOs producer on chestnut (Osmonalieva *et al.*, 2001). Therefore, only *P. leptostromiformis* is considered in this review.

Phomopsins are a group of mycotoxins capable of binding tubulin (Tonsing *et al.*, 1984) and causing the animal liver disease known as lupinosis (Allen and Wood, 1979; Gardiner, 1975a; Van Warmelo *et al.*, 1970a) which results in disorientation, blindness, lethargy and eventually death. It also results in significant production losses without any other obvious symptoms. Any animal grazing lupin stubble may be affected, but sheep are most sensitive (Seymour, 2009).

Lupin products are also increasingly being introduced into food for human consumption. The range of possible applications for lupins as a constituent in human nutrition is basically the same as for soybeans. Lupin flour can be added to bread, biscuit, pasta (ANZFA, 2001; Hill, 2005). Lupin hulls are also used as a fibre component in high fibre white bread and muffins (ANZFA, 2001). Lupin seeds can be employed for the production of plant-milk or different fermented products such as sauces or lactic acid beverages and yoghurts (Feldheim, 1999). All these derived products may be a source of human exposure to PHOs.

***Phomopsis* and its hosts**

The majority of *Phomopsis* species are characterised by the production of black pycnidial stromata, which can contain alpha-conidia (fusiform, biguttulate and hyaline) produced by uniloculate and multiloculate stromata, and beta-conidia (filiform, some tapered at one end) produced by small stromata. The teleomorph *Diaporthe* produces ascomata with asci clavate,

unitunicate and sessile containing ascospores hyaline, ellipsoidal and uniseptate (Goidanich, 1964; Williamson *et al.*, 1994a).
Phomopsis leptostromiformis produces both α and β conidia (Van Jaarsveld and Knox-Davies, 1974b; Williamson *et al.*, 1994a). In Western Australia *P. leptostromiformis* has been separated into two distinct morphological types designated biotype A (highly toxigenic) and biotype B (less toxigenic, forming ascomata in culture) (Wood, 1986; Wood and Sivasithamparam, 1989), later raised to varieties following extensive biochemical and cultural studies and named *P. leptostromiformis* var. *leptostromiformis* and *P. leptostromiformis* var. *occidentalis*, respectively (Shivas *et al.*, 1991).
Phomopsis has been recorded on lupins in Denmark (Lind, 1913), Poland (Kochman, 1957; Pape, 1927), Russia (Strukinskas, 1971), South Africa (Van Warmelo *et al.*, 1970a), USA (Ostazeski and Wells, 1960) and Australia (Ali *et al.*, 1982; Clarke and Kellock, 1979; Gardiner and Petterson, 1972; Stovold *et al.*, 1983; Wood and Brown, 1975; Wood *et al.*, 1973). *Phomopsis* was also found to be seed-borne in white and yellow lupines in USA (Ostazeski and Wells, 1960) and in Europe (Strukinskas, 1971) as well as in seeds of *L. angustifolius* in Poland (Nowicki, 1995).
More recently, in the 2007 growing season, elongated whitish-greyish patches covered with black stromatic conidiomata of *P. leptostromiformis* were described on lower stem parts above the soil on some yellow lupine plants in Sulejów, Poland (Marcinkowska, 2007), sometimes accompanied by sporodochia of *F. oxysporum* f.sp. *lupin*. Reappearance of the fungus in yellow lupine fields, especially on new Polish cultivars, might be a threat of *P. leptostromiformis* epidemic occurrence and the co-occurrence with *F. oxysporum* f.sp. *lupini*, suggests further studies on the relationship between the two fungi.
The discovery of ascomata of a *Phomopsis* led to the teleomorph described in culture and named as *D. woodii* (Punithalingam, 1974; Punithalingam and Gibson, 1975).
The teleomorph *D. woodii* is homotallic (Punithalingam and Gibson, 1975) and occurs naturally on infected lupin stubble in Western Australia (Wood and Brown, 1975). The first report of the fungus *D. woodii* on a *Lupinus* species was on *L. luteus* in Germany by Khün in 1880 (Fischer, 1893). *Diaporthe woodii* has been described as a stem pathogen infecting *L. luteus* (Kochman, 1957; Ostazeski and Wells, 1960), *L. cosentini* (Wood *et al.*, 1975) and *L. angustifolius* (Wood and Brown, 1975). It has also been reported to infect pods and seeds of *L. albus* (Van Warmelo *et al.*, 1970a; Wood and Petterson, 1985) and of *L. angustifolius* (Ali *et al.*, 1982; Clarke and Kellock, 1979; Petterson and Wood, 1986; Wood and Brown, 1975; Wood and Petterson, 1985; 1986). Leaf infection by *D. woodii* has been confirmed in Western Australia (Brown, 1984; Wood and Hamblin, 1981) even if lesions have only rarely been observed on lupin leaves during the growing season.
Diaporthe woodii has been isolated from lupin roots (Punithalingam and Gibson, 1975). In Western Australia, however, during the course of investigations of fungi associated with lupin root rots, it has rarely been detected on *L. angustifolius* (Wood and Sivasithamparam, 1989). Seed infection of *L. angustifolius* by *D. woodii* is widespread in Australia; 14 out of 20 seed samples from Victoria showed levels of infection between 2% and 12% (Clarke and Kellock, 1979) and 160 samples of commercial lupin seed from South Australia were infected up to 20% (Ali *et al.*, 1982).
In 1994, a distinct teleomorph for the toxigenic fungus, named *D. toxica*, has been proposed (Williamson *et al.*, 1994a). It has been isolated from *L. albus* in both eastern and Western Australia (Shankar *et al.*, 1995) and also in South Africa (Van Jaarsveld and Knox-Davies, 1974a). Cowley *et al.* (2010) showed that the isolate is capable of infecting all plant parts, is seedborne, and is reasonably distributed in the southern New South Wales cropping zone. They also demonstrated that under certain conditions *D. toxica* represents a significant threat to the Australian lupin industry.

Infection cycle

Diaporthe woodii remains viable in woody stem fragments for at least two years (Wood and McLean, 1982). Stromatal development starts soon after the appearance of stem lesions (Wood and Brown, 1975), then it differentiate into pycnidia and ascostromata.

In a field study managed in 1985 in Western Australia, α conidia were first detected in June (early winter) and continued to be liberated from the infected stubble of the previous season until November (late spring); ascospores were similarly released over the period July to November (Wood, 1986). Stylospores or β conidia of *P. leptostromiformis* have previously only been reported from South Africa (Van Jaarsveld and Knox-Davies, 1974a) from lupin stems incubated in the laboratory. However in Western Australia they were detected in field on lupin stems from the previous season as early as February (late summer) and continued to be released until August (Wood, 1986). It has been shown that inocula of α and β conidia and ascospores are all capable of infecting lupin seedlings in glasshouse experiments.

In Western Australia, the appearance of β conidial inoculum in the field on infected lupin stubble from the previous season is relevant for the survival of the fungus. As self-sown seedlings are commonly found in summer following rainfall from tropical cyclonic disturbances, β conidia from lupin stubble, either in the same paddock, or nearby, are then available to infect these seedlings.

Leaves of *L. angustifolius* seedlings have occasionally been observed in the field to have symptoms of infection by *P. leptostromiformis* (Wood and Hamblin, 1981). In Western Australia, symptoms of *D. woodii* infection in lupin stems do not usually appear before harvest. Brown (1984) found defoliated leaves of seedlings with advanced pycnidial development; as already noted, however, defoliation of a healthy crop does not commence until just prior to senescence. The role of leaf infection as a mechanism of secondary spread of the fungus within a crop is therefore not considered to be important.

Ecology

Phomopsis leptostromiformis can be readily isolated and cultured on either 1.5% malt extract agar with 100 μg/l of sodium novobiocin (Ali *et al.*, 1982; Van Warmelo *et al.*, 1970a) or potato marmite dextrose agar (PDMA)(Wood *et al.*, 1975; Wood and Petterson, 1985). On both media, a dense white surface mycelium is formed by biotype A with irregular stromatic masses developing in the myceliar layer after three weeks on a laboratory bench at 25°C. In contrast, biotype B isolates form a pigmented adpressed mycelial mat on PMDA with only sparse stromatal formation (Wood, 1986). The fungus can also be readily cultured on autoclaved lupin seeds, rape, sorghum, vetch, linseed and runner beans (Wood *et al.*, 1978) although in nature it has only been recorded on lupins. It can also be grown on liquid media (Lanigan *et al.*, 1979).

On senescent lupins stems in Western Australia biotype A produces dark purplish brown lesions which bleach with age and form both small and large black stromata; this fungus is isolated from field crops in 95% of cases. Biotype B forms bleached lesions, with minute stromata and prefers colonizing wild *L. cosentini* in more coastal areas of Western Australia (Wood, 1985; Wood and Sivasithamparam, 1989).

Shivas *et al*. (1991) showed that both biotypes produce PHO A and C. The biotype B was, therefore, considered to be not responsible for the lupinosis problem (Allen *et al.*, 1986; Wood and Sivasithamparam, 1989). Gardiner (1966) postulated the presence of an essential substrate in the lupin plant necessary for toxin production by the fungus. Wood *et al*. (1978) however, produced the toxin by culturing the fungus on a wide range of substrates, while Lanigan *et al*. (1979) produced the toxin also on a range of liquid media.

However toxin production on artificial media is temperature sensitive, with little toxin being produced above 28°C or below 22°C, even though there is little effect of temperature on growth within the range 20°C to 30°C (Wood and Sivasithamparam, 1989).
Diaporthe woodii saprobic nature is evident in the facility of isolation as a pure culture from infested stubble. Generally, lupin stubble heavily colonized by *D. woodii* are not toxic until they become moistened from either summer rainfall or a series of heavy dews (Allen *et al.*, 1980). If rain falls on the senescing crop just prior to harvest, however, toxicity can be present at harvest (Allen *et al.*, 1979). The precise conditions of moisture levels of the stubble, incubation period and field temperature necessary for toxin production are not known and warrant investigation. However after at least 10 mm of heavy soaking rain immediately followed by cloud cover with high relative humidity, toxin production in field stubbles has been known to produce symptoms of lupinosis in sheep in less than 48 hours (Wood and Sivasithamparam, 1989) and toxicity is stable, regardless of subsequent weather conditions (Allen *et al.*, 1979).

Plant-pathogen interaction
Phomopsis stem blight of lupins has many features of a latent disease (Wood and Sivasithamparam, 1989). Some studies (Kochman, 1957; Van Jaarsveld and Knox-Davies, 1974a) have been conducted into the early stages of infection on various lupin species, without reporting the mode of penetration or the means by which the fungus survives in symptomless plants as a latent infection. Williamson *et al*., (1991) reported for the first time the existence of subcuticular coralloid hyphae in lupin stems infected by *P. leptostromiformis*. Conidia of *P. leptostromiformis* do not form long germ tubes on narrow-leafed lupin stems, but germination is accompanied by the formation of a stain-absorbing swelling at the point of attachment to the cuticle. Penetration of the cuticle occurs directly below conidia through this thickened point of attachment. This unusual behaviour explains in part why previous workers (Kochman, 1957; Van Jaarsveld and Knox-Davies, 1974a) failed to detect the latent infection structure.
Field observations by Williamson *et al*. (1991) indicated that coralloid hyphae are rare in field-infected plants, but this is not surprising as not all plants show symptoms of Phomopsis stem blight at the end of the growing season, and some environments result in very little disease at all (Cowling *et al.*, 1988; Cowling *et al.*, 1987; Cowling and Wood, 1989). However, the observations on stubble provided the first evidence that normal colonizing hyphae grow directly from coralloid hyphae and invade the dead stem tissue upon senescence.

Physico-chemical characteristics and chemical analysis of phomopsins
Phomopsins A and B are macrocyclic hexapeptides (Allen and Hancock, 1989; Culvenor *et al.*, 1977) (Figure 1). Several derivatives of PHO A are known, namely phomopsinamine A and octahydrophomopsin A (PHO D). A third compound, PHO C, has been partially identified but a full structure is not yet available (Edgar, 1991).
Structurally, PHOs can be described as cyclic peptides presenting a 13-membered ring formed by an ether bridge in place of 2 hydroxyl groups (Edgar *et al.*, 1986; Mackay *et al.*, 1986); moreover, PHOs are characterised by several unusual amino acid residues, such as 3,4-didehydroproline, 2,3-didehydroisoleucine, 2,3-didehydroaspartic acid, 3,4-didehydrovaline, 3-hydroxyisoleucine and N-methyl-3-(3'-chloro-4',5'-dihydroxyphenyl) serine (Edgar, 1985; Edgar *et al.*, 1985; Edgar *et al.*, 1986).
Phomopsin A is soluble in water above pH 7.5 and below pH 1.0, reasonably soluble in aqueous alcohols, but only sparingly soluble in apolar media such as hexane (ANZFA, 2001**)**.
Very few analytical methods are currently available concerning the determination of phomopsins in food. In particular, since the occurrence of these compounds is only related to

lupin flour containing products, lupin seeds are the only food matrix considered in the literature. More specifically, two main methods have been reported for the determination of phomopsins in lupin seeds, being the former based on ELISA techniques (Than *et al*., 1992) and the latter on liquid chromatography separation followed by DAD and MS/MS detection (Hancock *et al*., 1987; Reinhardt *et al*., 2006).
Both the studies proposed a similar extraction procedure, performed by soaking the ground sample in a methanol-water (4:1, v/v) solution overnight at room temperature. After a homogenization step, the paste is stirred for 2 h and the supernatant containing the analytes is separated by centrifugation prior to analysis, without any specific clean up step (Than *et a*l., 1992; Reinhardt *et al*., 2006).
The chromatographic separation usually involved a gradient elution using methanol and *o*-phosphoric acid solution as eluents and a C18 analytical column. On account of the typical PHOs UV absorption maxima, methanol is used as a solvent medium (Culvenor *et al*., 1977), UV detection was performed at 210 nm and 290 nm (Hancock *et al.*, 1987; Reinhardt *et al*., 2006). Limits of quantification (LOQ) of 200 and 500 μg/kg have been reported for the HPLC detection of PHOs in lupin grain and stubble respectively (Hancock *et al.*, 1987).
Mass spectrometry can be used for both confirmatory and quantification purposes. In particular, an electrospray ionization (ESI) source operating in positive ion mode (ionization voltage: 5kV) was proposed by Reinhardt *et al.,* (2006): quantification was based on Multiple Reaction Monitoring (MRM) transitions, after a proper optimization of the collision-induced dissociation (CID) fragmentation parameters. This method showed a good sensitivity, with a detection limit (LOD) of 25 μg/kg for PHO A in cattle feed.
However, this method seems to not accomplish the requirements of Australian food safety authorities, since an upper limit of 5 μg/kg of PHOs in food destined for human consumption has been set.

Regarding immunochemical methods, ELISA assays are often used for the determination of these compounds in feed (Than *et al*., 1992; Allen *et al*., 1998; Than *et al*., 2005). In particular, Than *et al*., (1992; 2005) proposed an assay based on ovine polyclonal IgG antibodies, which were equally cross-reactive with both PHO A and its deschloro analogue PHO B. The sensitivity of the method is good, reaching a detection level of 1 μg/kg, thus accomplishing the Australian legal requirements. However, further studies should be done in order to assess the reliability of the method, in terms of ruggedness, accuracy and specificity, especially when very complex matrices such as cattle feed are considered.
Although both the HPLC and the ELISA methods have been implemented in the Australian food control laboratories, none of them has been validated by interlaboratory studies and there are no certified reference materials or proficiency studies available for the determination of PHOs.

Figure 1. The chemical structure of PHOs

Biosynthesis
The amino acidic sequence of PHO A has been established by heteronuclear 13C{1H} selective population inversion experiments and by fast atom bombardment mass spectrometry of PHO A and its derivatives; the structure was also confirmed by X-ray chrystallography (Culvenor *et al.*, 1989).
Several studies have been performed in order to investigate the chemical structure of PHO A and its derivatives (Cockrum *et al.*, 1994; Culvenor *et al.*, 1983; Culvenor *et al.*, 1978; Edgar

et al., 1985; Edgar *et al.*, 1986; Frahn *et al.*, 1983), but no specific investigation has been reported to date about biosynthesis.
To our knowledge, only one study reported some information about PHO analogues biosynthesis (Kobayashi *et al.*, 2003). In particular, the authors supposed that these compounds are biogenetically synthesized via a biological Diels–Alder reaction similar to the biosynthesis of solanapyrones and other decalin derivatives.
The PHO tripeptide side chain has been reported as crucial for the molecular interaction with tubulin (Mitra and Sept, 2004). For this reason, very recently, several studies have been reported about the enantioselective synthesis of this side chain (Grimley *et al.*, 2007; Ngo *et al.*, 2009; Ngo *et al.*, 2010; Shangguan and Joullie, 2009).

Occurrence data
The survey data available for PHOs are limited to Australian data and restricted to lupin seed only (Table 2). More recently a survey has been carried out on lupin seeds and flours, and on lupin-containing foods from the Swiss market (Reinhard *et al.*, 2006).
Australian surveys found that up to 20% of harvested seeds can be infected by *P. leptostromiformis* (Ali *et al.*, 1982; Clarke and Kellock, 1979; Wood and Petterson, 1985). Phomopsins were detected in 17 of 43 samples of lupin seeds from the 1981 harvest in three Australian states (Western Australia, Victoria and New South Wales). The PHO content, assessed by a nursling rat bioassay, ranged from <6 to 360 µg/kg (Petterson *et al.*, 1985). The extraction efficiency in these assays varied from about 15% at low concentrations (ca. 10 µg/kg), to 50-60% at high concentrations (40-60 µg/kg); therefore the values reported might be underestimated.
A detailed study has been done on *P. leptostromiformis* infection and PHO A content of seeds in a lupin crop having a high level of visible pod infection (Wood *et al.*, 1987). Over 96% of the visibly infected seeds contained viable *P. leptostromiformis*. Visually clean, or whole white seeds, from the primary pods had a higher level of fungal infection (56%) than similar seeds from the other pods (11%). The discoloured seed content of the primary pods was always higher compared to other pods. Although the cracked and discoloured seed fractions contained from 200 to 2,300 µg of PHO A/kg, the highest amount of this toxin detected in whole white seeds was 6 µg/kg.
In a survey of unsorted lupin seeds from the 1991-1992 harvests in Western Australia (Than *et al.*, 1994), the mean level of contamination by PHOs was 6.1 µg/kg (ELISA method, limit of detection of 1 µg/kg.); when seeds were sorted on the basis of discolouration, the mean level of PHOs in the clean seeds was 1.3 µg/kg, whereas, in the discoloured portions, the mean level was 355.1 µg/kg, with concentrations up to 4,522 µg/kg.
All lupin derived products can be contaminated by PHOs, but no investigations have been carried on the levels of PHOs in lupin flour. Therefore, it is not clear to what extent the milling process may remove PHO contamination. As it is known that PHOs are concentrated in the seed coat initially (Wood and Petterson, 1986), special attention needs to be directed towards gathering survey data for lupin hulls.
No data is available for other potential sources of exposure such as other lupin products, offal and milk. Moreover, no data is available on the levels of PHOs in the tissues of livestock, in milk or in other animal products (ANZFA, 2001).

Table 2. Results of surveys on phomopsins

Country	Commodity	Year	n[a]	LOQ (µg/kg)	*n* > LOQ	Mean (µg/kg)	Min/Max (µg/kg)	References
Australia	Lupin seed	1981	43	na	17	na	<6/360	(Petterson *et al.*, 1985)
Australia	Cracked and discoloured lupin seed	na	na	na	na	na	na/2,300	(Wood *et al.*, 1987)
	Whole white lupin seed	na	na	na	na	na	na/6	
Australia	Lupin seed non-sorted	1991/1992	88	na	na	6.1	nd/86	(Than *et al.*, 1994)
Switzerland	Lupin seed and flour	na	12	0.1	1	na	<0.1/20	(Reinhard *et al.*, 2006)
	Lupin containing foods		8	0.1	0			

[a]n = total samples
na : not available
nd : not detectable

Mitigation of phomopsins

Agricultural practices

Only a few strategies have been developed to limit contamination of lupin seed. In established lupin growing areas of western Australia, infection of *L. angustifolius* by *D. woodii* is endemic (Wood and Brown, 1975). Infested stubbles from previous crops invariably occur beneath subsequent ones, and due to the woody nature of the stems, retain infectivity even after two years of rotation (Wood and Sivasithamparam, 1989). This retention of trash occurs mainly as a consequence of the practice in Western Australia of minimum tillage to preserve soil structure (Jarvis *et al.*, 1986). Sanitation practices such as trash burying or burning can reduce the inoculum, but they are not well accepted in Australia because danger of erosion and degradation of soil structure.

The fungus can be introduced to new areas of lupin production through the use of infected seeds. However, the presence of asymptomatically infected seeds is almost always indicated by the concurrent presence of discoloured infected seeds (Wood and Sivasithamparam, 1989).

A further agronomic practice is the cropping of a susceptible lupin crop in combination with a non-susceptible host. This may be either a lupin cultivar resistant to the pathogen (Hamblin *et al.*, 1981; Wood and Hamblin, 1981) or a cereal such as oats (Wood and Sivasithamparam, 1989). As well as reducing the level of fungal infection of the susceptible lupin feed component, the practice of polyculture also provides the other non-infected feed component as a toxin-diluting factor.

The effect of calcium (Ca) supply on infection of narrow-leafed lupins (*L. angustifolius*) by *D. toxica*, has been investigated. Epidermal Ca concentration increased with increased Ca supply and there was an inverse relationship between the concentration of epidermal Ca and the frequency of infection by the fungus (Williamson *et al.*, 1994b).

The selection of resistant lupin cultivars

Considerable effort to control lupinosis in Australia has focused on the development of lupin cultivars resistant to Phomopsis infection and consequently with reduced levels of PHO in the stubble.

Some reports indicated that lupin species varied in resistance to Phomopsis stem blight, but few demonstrated variations in resistance within species. In field and glasshouse studies in

Poland, yellow lupin (*L. luteus*) was the only lupin species attacked by *P. leptostromiformis* (Kochman, 1957), but it was later reported that the fungus was highly aggressive to *L. luteus*, *L. angustifolius* and *L. albus* (Kochman and Kubicka, 1974). Yellow lupin was also highly susceptible according to a report from the USA (Ostazeski and Wells, 1960), but symptoms cannot be reproduced on *L. luteus* or *L. albus* without wounding and never on *L. angustifolius*.

In glasshouse and field experiments in South Africa, it appeared that *L. albus* is more susceptible than *L. angustifolius*, *L. luteus* and *L. mutabilis* Sweet, but symptom development do not vary among five *L. albus* cultivars, and similarly there is no variation among five *L. angustifolius* cultivars (Van Jaarsveld and Knox-Davies, 1974a).

In contrast, field studies in Australia showed that *L. albus* cultivar Ultra has very low levels of infection compared to *L. angustifolius* and *L. cosentinii*, cultivars all very susceptible (Wood and Allen, 1980). Recently, in south-eastern Australia, the incidence and severity of Phomopsis in *L. albus* crops increased and lead to further research on this disease (Cowley *et al.*, 2008).

A wild *L. angustifolius* line collected from Spain (P22750) was identified as resistant and used as a parent to produce the first resistant cultivars. Gungurru and Merrit (Cowling *et al.*, 1987). A breeding line 75A:258 has been shown as extremely resistant phenotype which can be traced to a wild parent from Morocco (P22872). Screening of F1, F2 and F3 families from crosses between 75A:258, Merrit and the susceptible cultivar Unicrop showed that 75A:258 has a single dominant allele Phr1 which confers resistance, whereas Merrit carries a different incompletely dominant resistance allele Phr2 (Shankar *et al.*, 2002). A co-dominant locus specific molecular marker has been produced based on an MFLP (microsatellite-anchored fragment length polymorphism) polymorphism linked to the Phr1 gene (Yang *et al.*, 2002) and a marker for Phr2 is under development. The European *L. albus* cultivars Ultra and Kiev Mutant and the *L. luteus* cultivars Teo and Motiv 369 had a good resistance to Phomopsis stem blight in Australia. Resistance reduced the lupinosis toxicity in stubbles grazed by sheep, even if variations in toxicity were not fully explained by variations in resistance or frequency of isolation of the fungus (Cowling *et al.*, 1988).

Resistance to infection of *L. angustifolius* by *D. toxica* has been shown as the result of an incompatible reaction between host and pathogen during the latent phase of the disease that appeared to occur after cuticle penetration. Resistant plants had a high frequency of small coralloid hyphae (10-80 µm length), while susceptible plants had a high frequency of large coralloid hyphae (80-400 µm length). Colonization of tissues below the cuticle began immediately after excision of stems from susceptible plants, but was delayed in resistant plants (Shankar *et al.*, 1998a). Conidial germination and penetration of the cuticle were not affected by host resistance, and resistance appeared to be the result of preexisting or induced resistance responses in epidermal cells below the infection site (Shankar *et al.*, 1996).

Breeders have been able to exploit natural epidemics and assessed resistance by rating the frequency and intensity of stem lesions on stubble immediately prior to the crop being harvested (Cowling *et al.*, 1987).

Williamson *et al.* (1991) developed an artificial inoculation, tissue staining and microscopic examination procedure to visualise subcuticular coralloid mycelia. Shankar *et al.* (2002) developed a non-destructive glasshouse test, based on inoculating one of the branches of the plant with a conidial suspension, while the other branch was protected from infection and maintained for seed production. An ELISA test, based on polyclonal antibodies to *D. toxica*, was also developed to distinguish large and small pathogen biomass as an alternative to microscopic examination (Shankar *et al.*, 1998b).

Cowley *et al.* (2008) developed and refined a screening assay using detached leaves and pods to screen for resistance to *D. toxica* in *L. albus*. The pod assay allows a better control of

experimental conditions and ensures that all pods being tested are at a similar physiological stage. In contrast with pods, Phomopsis infection in leaves is not considered to be an agronomically important character in albus lupins, but may play a role in epidemics (Brown, 1984). This method of assay has been undertaken to ascertain if foliar symptoms correlate with either pod or stem resistance. Assessing the leaves rather than pods has appeal in that it enables rapid screening of genotypes, without the need to grow plants till maturity. The authors found significant differences between genotypes for both assays. The correlation between the two assays was only moderate, but significant; however, it only accounted for 13% of the variability.

Chemical and biological control

Only a few studies have been done on the chemical control of *Phomopsis* on lupins.

In a trial managed in 4 successive years in Western Australia, plots of blue fodder lupin (*L. cosentini*) were sprayed with either water or solutions of 4 fungicides (Benomyl, Thiabendazole, Folpet, Captafol) or these treatments were also used together with a soil fumigation with methyl bromide. Results of fungicide sprays varied from failure in 1969-70 to partial control of lupinosis in 1970-71. Fumigation with methyl bromide was effective, but none of the fungicides could be considered practical for controlling lupinosis (Wood *et al.*, 1975). Moreover, in view of the extended infection period of lupins, from the seedling stage until senescence, the number of fungicide applications necessary to achieve control would not be economic. Captafol and methyl bromide are no longer permitted in Europe and other compounds are not specifically authorised in lupins.

The effectiveness of treating *L. angustifolius* stubbles with sodium hydroxide to kill the fungus *P. leptostromiformis* was evaluated in 1986 and 1987 (Croker and Allen, 1990), but the treatment did not significantly reduce the presence of living *P. leptostromiformis*.

No examples of microbial biocontrol are available in literature. A preliminary field experiment showed that lupin plots inoculated with lupin stubble infected with biotype B *L. leptostromiformis* reduced the level of natural infection by biotype A (Wood, 1986). Further work on biotypes could include attempts at biological control of toxigenic biotype A strains, possibly using more aggressive strains of biotype B as cross-protecting agents. Basic knowledge on the effect of environmental conditions on the activity of biotype B is needed for its successful establishment on lupins (Wood and Sivasithamparam, 1989).

Decontamination

There are only few examples in literature of decontamination or detoxification of PHOs and they showed resistance to destruction by extensive processing, including cooking and fermentation (ANZFA, 2001; Cockrum *et al.*, 1994).

Phomopsin A is hydrolysed and destroyed in solutions of sodium hydroxide. Two studies to evaluate the effectiveness of treating *L. angustifolius* stubbles with sodium hydroxide in the field to reduce the mycotoxins produced by *P. leptostromiformis* were carried out in 1986 and 1987 (Croker and Allen, 1990). Treatment of the stubble with a sodium hydroxide spray at rates between 6 and 18 kg/100 kg of stubble material did not significantly reduce the PHO A concentrations. In another study managed in 1984 (Allen *et al.*, 1986), when PHO levels were high, the PHO A concentration was lower for the samples obtained after the 2 highest rates of application (12 kg NaOH/100 kg with 1,200 or 4,800 l of water/ha), but these levels were not considered safe for grazing sheep. The authors dismiss the use of higher concentrations of NaOH as impractical and dangerous. It remains to be seen whether NaOH applied as a spray on lupin stubbles after harvest can achieve similar results.

Instead, seed sorting can be an effective mean of reducing PHOs seed contamination. Data reported by Than *et al.* (1984) demonstrated that the highest levels of PHOs are present in the

discoloured lupin seeds and confirmed the suggestion that removal of the discoloured seeds by commercial grading provides a ready means of ensuring the safety of lupin seed (Wood and Petterson, 1986; Wood *et al*., 1987).

Pharmacokinetics of phomopsins

No information was found on the absorption, metabolism, distribution or excretion of PHOs. *In vitro* studies using artificial rumen preparations suggest that PHOs do not undergo any significant metabolism by rumen microorganisms (Peterson, 1986). After high levels of ingestion, the toxicity of PHOs may be limited by its rate of absorption (Peterson, 1986), as suggested by the differences between oral and subcutaneus (sc) or intraperitoneal (ip) LD_{50}s (see following section). Cytotoxic effects in liver were observed one hour after ip administration of PHOs to rats (Peterson, 1978), suggesting that once absorbed, PHOs may undergo very rapid transport to the liver, probably through the portal vein. There is limited evidence, from kidney effects observed in toxicity studies, that PHOs, or an active metabolite of PHOs, may be excreted via the kidneys (Peterson, 1986; 1990; Peterson and Lanigan, 1976).

Toxicology of phomopsins

A comprehensive review of the toxicology of PHOs was published in 2001 by the Australia New Zealand Food Authority (ANZFA, 2001). Since then, less than a handful of publications on this subject have appeared in the literature.

Acute toxicity

Acute toxicity studies have been carried out in mice, rats and sheep. In all three species, the primary organ of toxicity is the liver, with liver failure being the most plausible cause of death. Sheep appear to be the most sensitive species (based on comparisons of PHOs toxicity after sc injections). Oral toxicity was determined in rats and sheep, and appeared to be significantly lower, suggesting a low absorption of PHOs after oral administration. PHOs bind to tubulin (Tonsing *et al.*, 1984), and this binding is believed to be responsible for their toxicity. Indeed at submicromolar concentrations, PHOs prevent the polymerisation of tubulin (Lacey *et al.*, 1987; Ngo *et al.*, 2009; Peterson, 1990). This interaction inhibits spindle formation during mitosis, and leads to mitotic arrest. When this occurs *in vivo* in hepatocytes, it is followed by cell death (Peterson and Lanigan, 1976), suggesting that the result of the binding of PHOs to tubulin are irreversible, though studies in cultured cells have provided conflicting results (Brown and Bick, 1986; Tonsing *et al.*, 1984). PHOs have also been reported to affect cell membranes, with changes in the activity of some membrane-associated enzymes, increased fluidity, and redistribution of Golgi apparatus membranes (Tonsing *et al.*, 1984; Peterson, 1986).

Mice. Mice were injected ip with crude preparations of PHOs, however, the exact dose of PHOs was not reported (Papadimitriou *et al.*, 1974; Peterson and Lanigan, 1976). The liver was the main target organ of toxicity. Histological changes, as well as changes in the activities of various hepatic enzymes were observed. Such changes persisted for several days but gradually returned to normal after four weeks. An increase in the number of parenchymal cells undergoing mitosis in the liver was seen after ~24 hours, which reached a plateau between 48 and 72 hours, and then declined. Similar mitotic effects were also seen in the kidneys, though later than in the liver, but not in other organs.

Rats. LD_{50} values for PHOs in rats were reported by Peterson (1986). Oral LD_{50}s were 24 - 52.5 mg/kg, while sc and ip LD_{50} values were much lower, 4.4 - 8.0 mg/kg, and 1.2 - 2.0

mg/kg, respectively. In another study, young male rats (two weeks old) were given a single ip dose of a crude preparation of PHOs containing approximately 4% toxin on a dry weight basis (Peterson, 1978). The calculated doses were between 0 and 2.64 mg/kg. Nursing rats were selected over adult rats for this experiment, as they were expected to exhibit a high level of mitotic activity, and hence to be a better model for studying the effects of PHOs on cell cycle. Animals were sacrificed at various intervals (up to 28 days) after injection. The LD_{50} of PHOs in this study was estimated to be about 1 mg/kg, with deaths occurring within 4 to 8 days. This LD_{50} value is consistent with values in adults, and suggests a lack of age-related sensitivity to acute PHOs toxicity. The main effect at low PHOs doses (<0.04 mg/kg) was metaphase arrest in liver parenchymal cells, which was observed within one hour from injection, reached its peak at 2-4 days, and then declined. Mitotic arrest was also observed in the kidney and pancreatic acinar cells, but only at high doses (0.7 mg/kg for kidneys and 1 mg/kg for the pancreas), and not in any other tissues. At higher dose levels (>0.17 mg/kg), fatty changes and fibrosis developed in the liver, reaching a maximum at 3-4 days, and the rats became jaundiced. Depletion of cortical cells in the thymus, depletion of haematopoietic tissue in the spleen, reduced gastric activity, and retarded growth rates were also observed.

Sheep. In sheep, the ingestion of PHOs-contaminated lupin stubble is associated with the occurrence of lupinosis. The classical signs of lupinosis are inappetence, weight loss, lethargy and jaundice (Gardiner, 1975; Gardiner and Parr, 1967; Van Warmelo *et al.*, 1970). In acute lupinosis, there is usually jaundice, and the liver is greatly enlarged. To quantify acute toxicity, male sheep were given a single sc dose of PHOs at doses of 1.25 – 98 µg/kg (Jago *et al.*, 1982). Sheep given ≥ 75 µg/kg died within 3-5 days, while those given between 10 and 37.5 µg/kg died in 10-26 days. All sheep receiving 5 µg/kg or less, survived the 28 days of the experiment. The first sign of toxicity was inappetence, which progressed to anorexia. In sheep given lethal doses of PHOs, both total serum protein and albumin levels decreased by 10-17% within 4 days, with albumin levels continuing to decline, consistent with a diagnosis of liver failure. Given the limited number of animals used in this study, an exact LD_{50} could not be established, but survival time was inversely related to dose level. A rough estimate would place the LD_{50} value between 5 and 10 µg/kg, indicating that the sheep is much more sensitive to PHOs toxicity than rodents.
In another study in this animal species, sheep were administered single doses of PHOs by either the sc or the intraruminal (ir) routes (Peterson *et al.*, 1987). The ir injection was used to simulate the retention of plant material in the rumen and is considered equivalent to oral administration. Decreased appetite was observed in 1/3 sheep administered a single sc dose of 2.0 µg/kg and appetite was completely suppressed in 3 sheep given a single sc dose of 10 µg/kg, with two animals dying 4 days after exposure. Even in this study, the approximate LD_{50} of PHOs in sheep after sc administration was approximately 10 µg/kg bw. A single ir dose of 1 mg/kg caused the same clinical, biochemical and histological effects as a single 10 µg/kg sc dose, confirming poor gastrointestinal absorption of PHOs. A single ir dose of 0.5 mg/kg caused significant liver damage, but no deaths. Lower single ir doses (0.125 and 0.250 mg/kg) did not cause any detectable tissue damage, but were associated with loss of appetite.

Sub-Chronic toxicity
Rats. Phomopsins was given by sc to 10-week old Long-Evans rats of both sexes at the dose level of 30 µg/kg bw, 5 days/week for 2, 6 or 17 weeks (Peterson, 1990). Animals were sacrificed at scheduled intervals during treatment period, and up 6 months or 2 years after treatment. No clinical or behavioural signs were observed during treatment. Survival time was decreased in rats treated for 6 and 17 weeks. All rats given PHOs for 17 weeks developed

irreversible liver damage, characterised by nodular cirrhosis and extensive biliary hyperplasia, which continued to progress after the treatment ceased. In contrast, in some rats treated for only six weeks, an almost complete regression of liver and biliary tract lesions was observed, with only a small amount of fibrous tissue evident two years after the last PHOs administration. Livers of rats given PHOs for two weeks also exhibited full recovery within a few weeks after the end of treatment.

An interesting aspect of this study is that the incidence of tumours at death, up to two-years of age, was examined. Tumours present in PHOs-treated rats were localized in the liver, and comprised cholangiomas, cholangiosarcomas and hepatocellular carcinomas. Incidence of tumors increased with increasing treatment duration (Table 3).

Table 3. Incidence of tumours in rats following subcutaneous administration of phomopsins (30 µg/kg bw/day for the indicated period).

Treatment Duration	No. of animals	Cholangioma	Cholangiocarcinoma	Hepatocellular carcinoma
Untreated	59	0	0	0
2 weeks	20	0	0	0
6 weeks	34	10	1	0
17 weeks	37	22	2	3

Adapted from ANZFA (2001).

Sheep. Male sheep were administered multiple doses of PHOs by the i.r. route (Peterson *et al.*, 1987). Increased toxicity was observed when a total dose of 1 mg/kg bw was given in multiple lower doses of 50 or 200 µg/kg, as compared to the same dose given as a single injection. This again suggests that absorption of high dose levels PHOs from the gastrointestinal tract is limited and saturable. The main effects of PHOs exposure were observed in the liver. Histopathological examination revealed fatty changes which progressed to fibrosis, with a variable degree of proliferation of biliary tissue. Pigmented macrophages containing ceroid and haemosiderin, were abundant in the fibrous tissue.

Cows. Holstein-Friesian cows in mid-lactation were dosed orally with PHO A (2.88 mg/cow/day, corresponding to 5.2 µg/kg bw/day based on an initial average body weight of 556 kg) for eight weeks (Hough and Allen, 1994). Cows were returned to pasture for four weeks after the exposure period before cessation of the study. Milk yield, milk composition, body weight and body condition were measured weekly for the eight week treatment period and for four weeks after treatment. Liver damage was monitored by the measurement of plasma γ-glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH) activities, and plasma bilirubin concentrations were determined weekly during the eight week treatment period. No measurable effects on milk yield, fat, protein or total solids content were found. No significant differences in cows was found with respect to body weight, plasma GGT and GLDH activities or bilirubin concentration.

Chronic toxicity

There are no chronic toxicity studies in any animal species for PHOs. Observations in sheep affected by lupinosis indicate liver alterations, and watery lumen content, with very little solid matter in the abomasum and the small intestine. The caecum contains hard, dry, impacted faecal material. Ascites may be present, and there is evidence of general muscle wastage (Gardiner, 1965; 1967).

Reproductive and Developmental toxicity

Peterson (1983) examined the effect of PHO A on pregnant rats and their embryos. In a first experiment, pregnant rats were injected ip with 30 or 90 µg/kg bw/day PHOs, on gestational days (GD) 6-10 or 11-15. In a second experiment, 25, 100, or 400 µg/kg of PHOs were administered, as a single ip dose, to pregnant rats on GD 6, 8, 10, 12 or 14. In both experiments, rats were sacrificed on GD 20 for examination of foetuses. The dose of 90 µg/kg bw/day caused death of 40% of the dams. Liver damage was observed in all dams treated with PHO A, regardless of the dose or the duration of exposure. A single dose of 400 µg/kg or a dose of 90 µg/kg bw/day for 5 days, were associated with high embryo lethality. The dose of 30 µg/kg bw/day for 5 days was embryo-lethal only when administered on GD 6-10. Foetuses surviving the dose rate of 90 µg/kg bw/day had severely retarded growth and presented irregular skeletal ossification. Of interest is that the livers of the foetuses were apparently unaffected, and there was an absence of metaphase arrests in all embryonic tissues examined. This would suggest that the embryonic deaths may not be associated with direct PHO A action on their tissues, but may instead be the indirect result of maternal toxicity. No studies on the reproductive toxicity of PHOs were identified.

Genotoxicity and Carcinogenicity

Limited information is available on the genotoxicity of PHOs. Results in the Ames test, and in the Chinese hamster ovary chromosome aberration and HGPRT locus mutation tests, were negative (BIBRA, 1986). In contrast, PHO A was shown to induce chromosomal aberrations consisting of chromatid and isochromatid deletions and chromatid exchanges in the Chinese hamster DON cell line (Brown and Bick, 1986).

No long-term carcinogenicity study has been carried out with PHOs. However, the sub-chronic study discussed in a previous section indicates that administration of 30 µg/kg/day PHOs to young rats for 6-17 weeks causes the development of hepatic tumours (Table 3).

Legislation on phomopsins

Australia and New Zealand are the only countries to include PHOs in their mycotoxin regulations, with a limit of 5 µg/kg in lupin seeds and products of lupin seeds (van Egmond and Jonker, 2004). This appears to be due to the emergence of lupin flour as a human food ingredient and the availability of data on the PHO content of lupins in Australia (ANZFA, 2001; Petterson *et al.*, 1985; Than *et al.*, 1994; Wood and Petterson, 1986). In fact, in 2002 New Zealand and Australia initiated a joint food regulatory approach, codified in the Australia New Zealand Food Standards Code. Prior to drafting of the joint Code, a series of risk assessments were carried out by Food Standards Australia New Zealand (FSANZ; later called Australia New Zealand Food Authority). This reported a review of the maximum permitted concentrations of non-metals in food, including some mycotoxins.

Conclusions and research needs

Phomopsis leptostromiformis, and its teleomorph *D. toxica,* has been reported to cause stem blight in young lupins and to produce PHOs. These mycotoxins are capable of binding tubulin, causing the animal liver disease known as lupinosis. Limited information is available on fungal ecology, plant-pathogen interaction and mitigation strategies.

ELISA and HPLC based analytical methods are reported, but none of the mentioned methods has been validated by interlaboratory studies, and there are no certified reference materials or proficiency studies available for the determination of PHOs.

Surveys available for PHOs occurrence are limited to Australian data, and restricted to lupin seed. No data are available on food and feed products, though risks for their contamination clearly exist. No information is also available regarding the presence of PHOs in animal-derived products.

Given the paucity of available data, the assessment of potential risks related to exposure to PHOs is not currently feasible. Research efforts are strongly suggested in all areas discussed in the present review. Validated analytical methods are a necessary starting point, before planning any surveys finalised to understand whether any concern related to PHOs in Europe is justified. PHOs show clear toxic effect on the liver, but a better knowledge on their mechanism of action, and particularly on dose-effect relationships, are necessary to define tolerable daily intake levels. A better knowledge of the pathosystem and the role of ecological and agricultural parameters will be needed to support prevention strategies, which are of crucial importance for these and other mycotoxins, given the poor role played by decontamination strategies.

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