National Diagnostic Protocol
Lentil Anthracnose
(*Colletotrichum truncatum*)

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DISCLAIMER

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1.0 Introduction

The causal pathogen of lentil anthracnose (*Colletotrichum truncatum*) has an extensive distribution worldwide, including Australia. The disease, lentil anthracnose, has been recorded worldwide in several lentil-producing countries including Bangladesh, Bulgaria, Brazil, Pakistan, Ethiopia, Morocco, Syria, the USA and Canada. Hosts of *C. truncatum* include not only *Lens culinaris* (lentil), but also other leguminous hosts including species of *Vicia*, *Vigna*, *Phaseolus*, and several specific crop species including *Pisum sativum* (field pea), *Arachis hypogea* (peanut) and *Glycine max* (soybean). The discovery of the disease in Manitoba, Canada in 1987, led to lentil anthracnose being recognised as an economically important disease of lentil.

In Australia, *C. truncatum* has not been observed on lentil under field conditions, despite extensive crop surveys and seed testing. However, *C. truncatum* has been identified on other host species including peanut and soybean, generally in northern cropping areas of New South Wales and parts of Queensland. Scientific literature suggests the existence of host specific pathotypes within the pathogen species. In Canada, the lentil attacking form of *C. truncatum* can also infect faba bean (*Vicia faba*) and wild vetches (*Vicia* spp.), and has been shown to infect field pea in glasshouse testing (Morrall *et al.* 1989). There is no evidence that the lentil-attacking form of *C. truncatum* is present in Australia.

The anthracnose pathogen survives between growing seasons primarily on infected stubble. However, microsclerotia, which are formed within stem lesions, can persist freely in the soil and remain viable for up to four years. Infection is initiated within the crop by infected stubble or microsclerotia coming into contact with lentil seedlings. Leaf infection follows soon afterwards, which later develops into stem lesions. Spores formed within lesions are splash dispersed by rain droplets to cause secondary infections on surrounding plants. When infected crops are harvested, small fragments of stem and pod tissue can be dispersed by wind to surrounding paddocks, which can initiate infection in subsequent lentil crops. Seed can become infected by the pathogen, but generally at very low levels. Seed to seedling transmission of the disease has not been observed but cannot be discounted as a pathway of disease introduction.

In Canada, lentil anthracnose is managed through a combination of crop rotation, tillage to encourage breakdown of infected stubble and microsclerotia and the use of foliar fungicides. Crops left unprotected can suffer yield losses of up to 60% (Morrall 1997).

International disease nurseries have shown that current Australian lentil cultivars are highly susceptible to lentil anthracnose. This disease poses a serious threat to the Australian lentil industry, and is ranked by Plant Health Australia as the highest fungal threat to lentil, based on the knowledge of scientists and industry.

2.0 National Diagnostic Protocol Procedure

2.1 Purpose and scope of diagnostic protocol

The purpose of this manual is to provide a nationally accepted standardised protocol for the accurate identification of the lentil-attacking form of *Colletotrichum truncatum*, the causal pathogen of lentil anthracnose. The lentil-attacking form of *C. truncatum* is a quarantinable pathogen in Australia, but it is found in many lentil producing countries throughout the world including Bangladesh, Bulgaria, Canada, Ethiopia Pakistan, Syria and the USA.

The pathogen *C. truncatum* has already been identified in Australia on other host species including peanut (*Arachis hypogea*) and soybean (*Glycine max*). Within the species of *C. truncatum*, host specific pathotypes are known to occur. In Australia, the pathogen has never been observed in the field on lentil, which suggests that the lentil-attacking pathotype is not in Australia. Limited morphological variation among *C. truncatum* from different host species makes identification at the sub-species level difficult. Given these morphological similarities there is a need for a rapid, accurate method of identification of the lentil-attacking form of *C. truncatum*.

This manual is designed to be a complete guide to the identification of the lentil-attacking form of *C. truncatum*. This includes a Pest Risk Analysis for *C. truncatum*, sample collection and diagnostic techniques for identification of the lentil-attacking form of *C. truncatum*. Colour plates of symptoms in the field and key references are included.

2.2 Responsibility

Figure 1 shows a flow diagram of the responsibilities and procedures required when a suspect sample is received. The responsibilities are also listed clearly in the following points:

A: State/territory agriculture departments receiving suspect plant sample:

- Receiving scientists will record details of the sample so that a trace back can occur if required.
- Receiving scientists will examine the sample and provide diagnostic services (in this case, conducting the morphological identification and/or PCR test) to identify the pathogen.
- Receiving scientists will notify the State Quarantine Authority (eg. DPI-Victoria Plant Standards Branch) of the suspect sample.
- State Quarantine Authority will examine the evidence and inform Office of the Chief Plant Protection Officer (OCPPO) and AQIS and advise scientists of required action.
- The State Quarantine Authority will participate in the Consultative Committee on Exotic Plant Pests and Diseases (CCEPPD), chaired by the Chief Plant Protection Officer and decisions made and actions required will be passed onto state scientists for action.

- Scientists may be requested to provide expert advice to the CCEPPD.
- Scientists will undertake procedures as advised by the State Authority eg. Conducting a second type of diagnostic test (secondary confirmatory test) and sending part of the sample to the interstate confirmatory laboratories for repeat of the primary diagnostic test.
- Under direction from the State Authority, state scientists will undertake delimiting surveys if required and undertake diagnostics on survey samples.
- The State Authority will liaise with industry representatives.
- The State Authority will develop communication strategies in conjunction with the CCEPPD.
- The State Authority will report to all interested parties (OCPPO, CCEPPD, AQIS, national bodies and industry) as required.
- The State Authority will keep up to date with the processing of the suspect sample and will notify the clients of the final result and the corresponding decision for that result.
- The State Authority will handle all correspondence with clients. This is very important and is to be made clear to other personnel involved with handling the sample that they are not to correspond with the client.

B: Interstate agriculture departments

- Scientists will re-examine the suspect sample.
- Scientists will repeat diagnostic tests and confirm diagnosis.
- Scientists may be requested to provide expert advice to the CCEPPD.
- State Quarantine Authority will inform the Chief Plant Protection Officer and the CCEPPD and will implement their decisions.

C: Office of the Chief Plant Protection Officer (OCPPO)

- OCPPO will convene the CCEPPD and all decisions regarding the steps involved in handling and diagnosing the original sample will be made by the committee.
- The CCEPPD will determine whether or not the incursion requires a national response or involves only one state and will determine the need for delimiting surveys.
- Information from each state will be provided to the CCEPPD to enable national decisions to be made.
- OCPPO will provide media releases to the public and interested parties.
- OCPPO and the CCEPPD will determine whether or not the pathogen can be eradicated, contained or will be declared endemic.

2.3 Procedure

Direct examination of symptoms and fungal cultures is not always a reliable method. Due to morphological similarities between isolates of *C. truncatum* from different host species, identification of the lentil-attacking strain of *C. truncatum* has to be confirmed by molecular techniques such as conventional PCR.

2.4 Documentation

An electronic and a hard copy of this manual are maintained and updated by the Pulse Pathologist, Primary Industries Victoria (PIRVic), Department of Primary Industries – Horsham, Victoria.

2.5 Records

The recording sheets contained in the Appendices must be copied and filled in as appropriate for each sample received and kept together in a file marked „Suspect Lentil Anthracnose Samples". All documents must be also be copied and sent to the confirmatory laboratory if the initial processing of the sample is conducted by the „State Laboratories".

Any data relating to the validation of a method must be kept for as long as the method is in use.

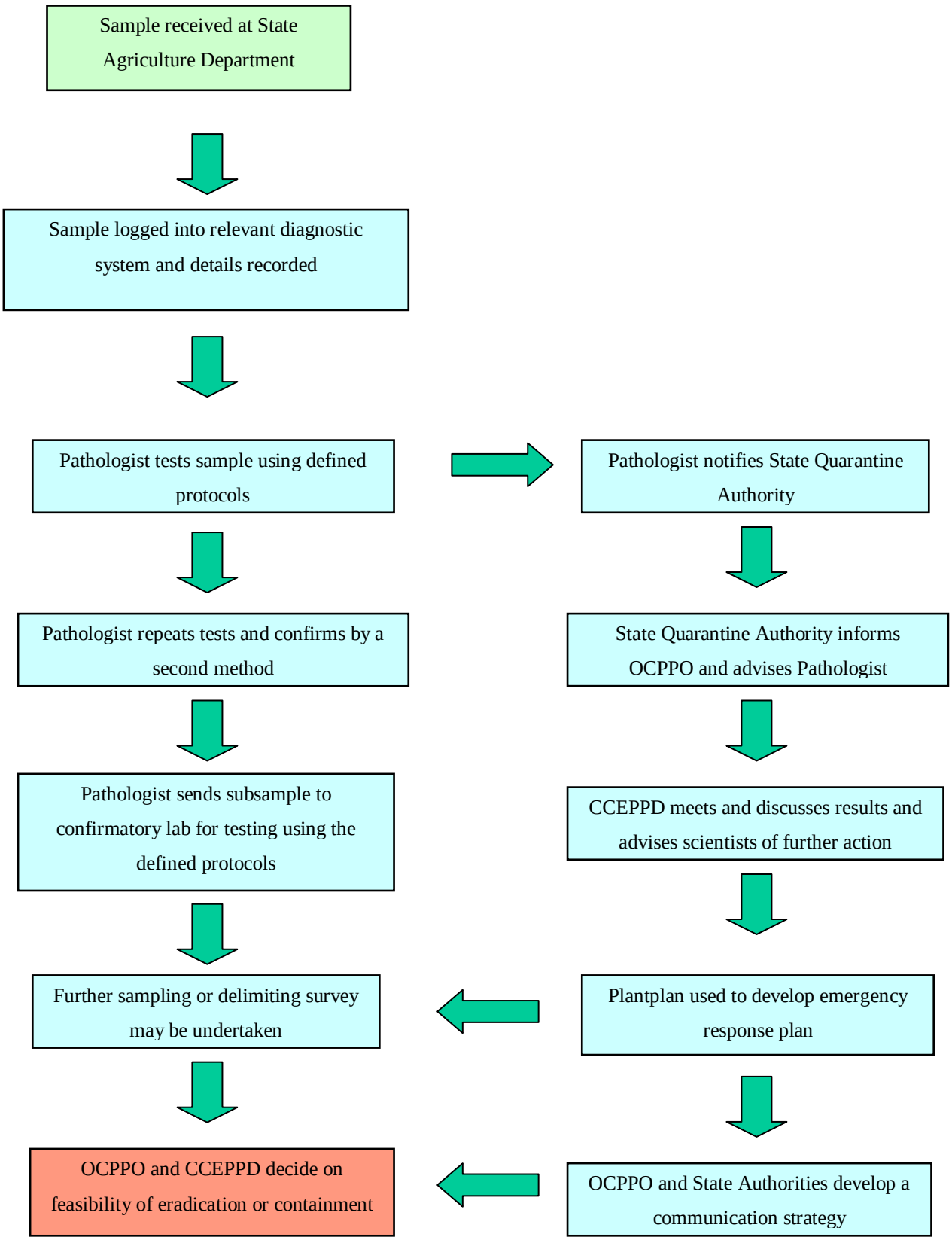

Figure 1. Flow chart of the basic procedure and responsibilities of the relevant Departments if a suspect sample is received.

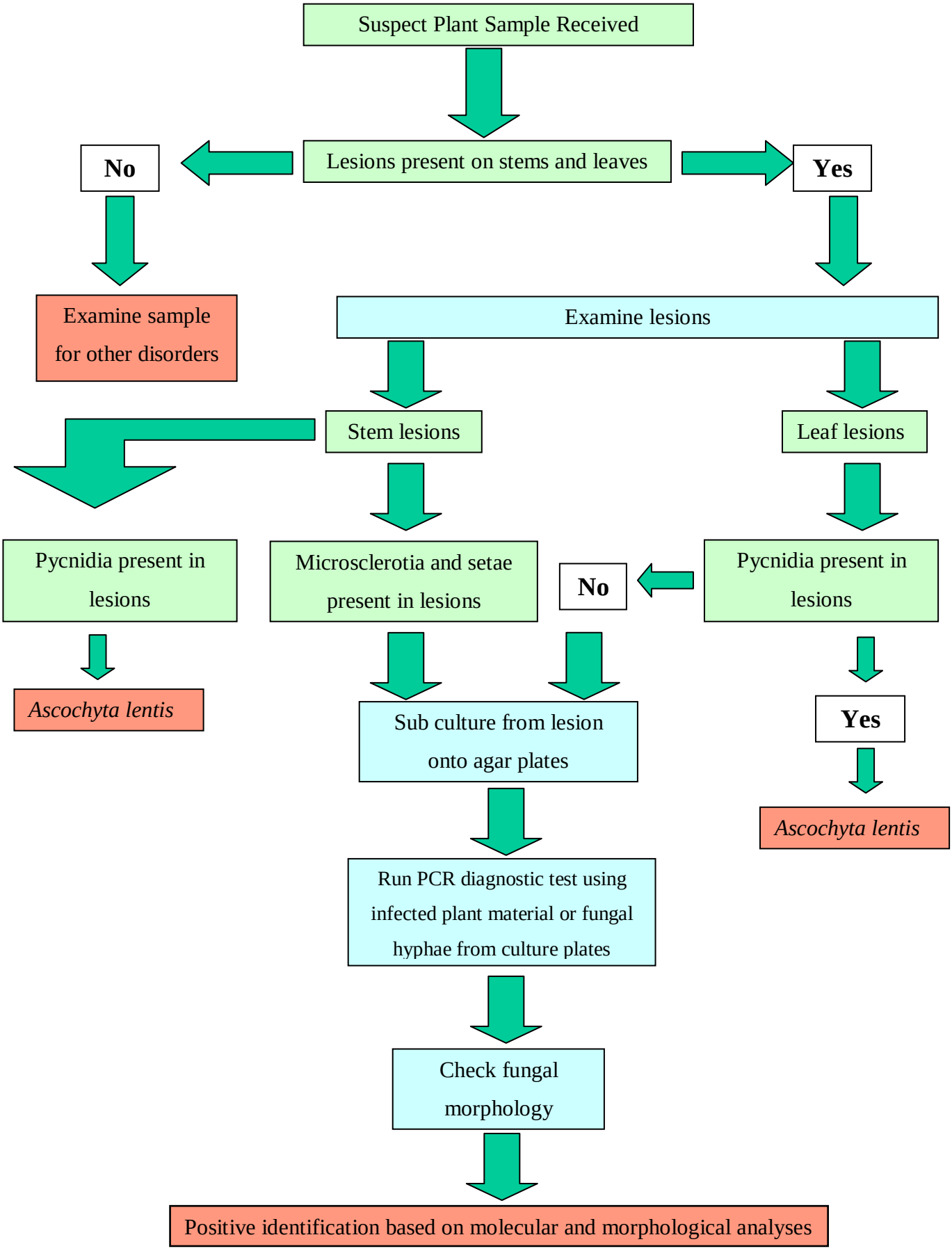

Figure 2. Flow diagram of the protocols for the analysis of a suspect plant sample

3.0 Pest Risk Analysis

3.1 Background

Lentil anthracnose (*Colletotrichum truncatum)* is listed on the Australian Quarantine and Inspection Service (AQIS) ICON Import Conditions database as a quarantinable pathogen of lentils in Australia. Lentil plants are examined for lentil anthracnose in post-entry quarantine as required in the regulations listed on ICON.

3.2 Species name

Colletotrichum truncatum (Schwein.) Andrus & W.D. Moore, 1934

3.3 Synonyms or Changes in Combination or Taxonomy

Colletotrichum dematium forma truncatum (Schwein.) Arx, *Vermicularia truncata* Schwein. 1832

3.4 Common names

Brown blotch of cowpea, colletotrichum leaf spot of moth bean, lentil anthracnose, pod blight of soybean, soybean anthracnose, stem anthracnose of lima bean and pigeon pea

3.5 Host range of *C. truncatum*

Host	**Reference**
Abutilon theophrasti (chinese lantern)	CAB International (1999)
Adiantum sp. (maidenhair fern)	Anonymous (1985 onwards)
Aeschynomene americana (glen joint vetch)	United States Department of Agriculture (2000)
Apocynum cannabinum (Indian hemp)	United States Department of Agriculture (2000)
Arachis hypogaea (peanut)	Anonymous (1985 onwards), United States Department of Agriculture (2000)
Cajanus cajan (pigeon pea)	United States Department of Agriculture (2000)
Capsicum annuum (green pepper)	CAB International (1999)
Chenopodium album (pigweed)	United States Department of Agriculture (2000)
Clitoria ternatea (butterfly pea)	United States Department of Agriculture (2000)
Datura stramonium (common thorn apple)	United States Department of Agriculture (2000)
Desmodium sp.	United States Department of Agriculture (2000)
Glycine max (soybean)	Anonymous (1985 onwards), United States Department of Agriculture (2000)
Ipomoea sp. (bindweed, sweet potato)	United States Department of Agriculture (2000)
Lens culinaris (lentil)	Agarwal & Prasad (1997), Morrall (1997), United States Department of Agriculture (2000)

Host	Reference
Medicago sativa (lucerne)	United States Department of Agriculture (2000)
Panax ginseng (asiactic ginseng)	CAB International (1999)
Phaseolus spp. (beans)	Agarwal & Sinclair (1987), United States Department of Agriculture (2000)
Pisum sativum (field pea)	CAB International (1999)
Stylosanthes spp.	United States Department of Agriculture (2000)
Trifolium fragiferum (strawberry clover)	Shivas RG (1989)
Trifolium pratense (red clover)	CAB International (1999)
Trifolium subterraneum (subterranean clover)	Shivas RG (1989)
Vicia angustifolia (narrowleaf vetch)	United States Department of Agriculture (2000)
Vicia atropurpurea (purple vetch)	United States Department of Agriculture (2000)
Vicia faba (broad bean)	United States Department of Agriculture (2000)
Vicia pannonica (Hungarian vetch)	United States Department of Agriculture (2000)
Vicia sativa (common vetch)	CAB International (1999), Chandrashekar (1993), United States Department of Agriculture (2000)
Vicia spp. (vetches)	Neergaard (1979)
Vicia villosa (hairy vetch)	Anonymous (2000)
Vicia villosa ssp *dasycarpa* (woolly-pod vetch)	Anonymous (1985 onwards)
Vigna aconitifolia (moth bean)	CAB International (1999)
Vigna mungo (black gram)	CAB International (1999)
Vigna parkeri	Anonymous (1985 onwards), Chandrashekar (1993)
Vigna radiata (mung bean)	CAB International (1999)
Vigna sesquipedalis (asparagus bean)	Oliva *et al.* (1990)
Vigna unguiculata (cowpea)	Anonymous (1985 onwards), Chandrashekar (1993), CAB International (1999)
Xanthium occidentale	Anonymous (1985 onwards)

3.6 Distribution

3.6.1 Australian status

Colletotrichum truncatum is endemic on non-lentil hosts but the lentil-attacking pathotype is exotic.

3.6.2 Current distribution

Regions/Countries	Reference
Australia (NSW, Qld, WA, NT)	Anonymous (1985 onwards), Shivas RG (1989)
North America, South America, Central America, Africa, South Africa, Europe, Middle East, Asia, South East Asia, Oceania	Venette *et al.* (1994), Kaiser *et al*. (1998), CAB International (1999), United States Department of Agriculture (2000)

3.6.3 Potential distribution in Australia

The potential distribution of lentil anthracnose is considered to be those regions where the main host crop lentil is grown. In Australia this includes the Wimmera region of Victoria, and the South-east, Mid-north, Yorke Peninsula and Eyre Peninsula regions of South Australia. However, other leguminous crop species including faba bean and vetch can also host the lentil-attacking pathotype of *C. truncatum,* and are grown throughout the Australian grain belt (refer figure 3). Climatic conditions between regions in Canada where the disease already occurs and areas of Australia are also similar.

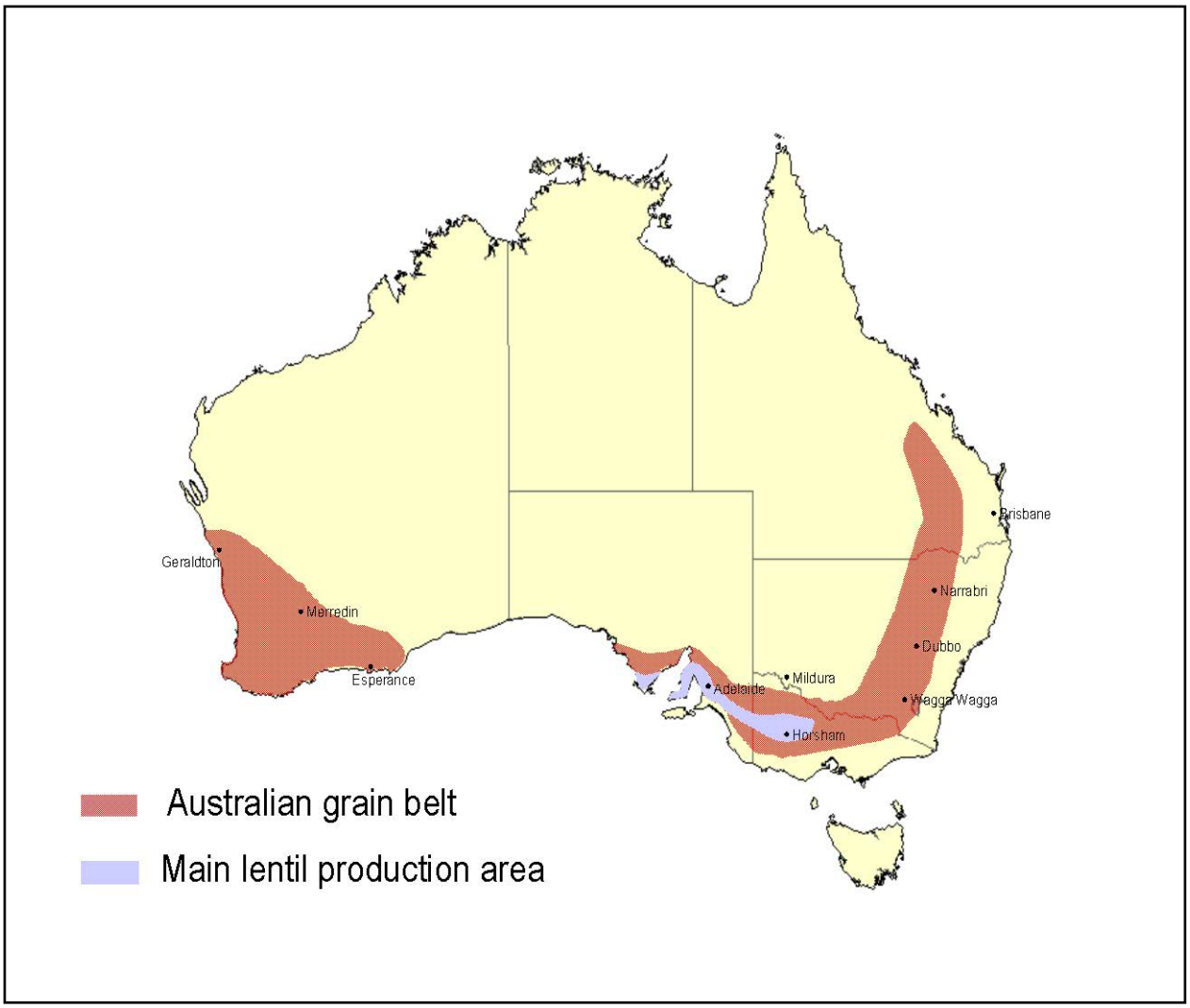

Figure 3. The potential distribution of lentil anthracnose covers the main lentil production areas of Australia.

3.7 Plant parts affected

Stems, leaves, inflorescence, pods, seed

3.7.1 Seedborne Hosts

Host Species	Reference
Lens culinaris (lentil)	Morrall *et al.* (1993), Agarwal & Prasad (1997), Morrall (1997), Kaiser *et al*. (2000)
Vicia spp.	Neergaard (1979)
Vicia villosa	Richardson (1990)
Arachis hypogea (peanut)	Olivia *et al*. (1990)
Glycine max (soybean)	Agarwal & Sinclair (1987), Olivia *et al.* (1990)
Phaseolus lunatus	Agarwal & Sinclair (1987)
Vigna sesquipedalis	Olivia *et al*. (1990)

3.8 Disease features

There are several common foliar diseases of lentil in Australia, these include Ascochyta blight (*Ascochyta lentis*) (see figure16) and Botrytis grey mould (*Botrytis cinerea* and *B. fabae*). Lentil anthracnose may be easily mistaken for one of these common foliar diseases, but there are several key symptoms that are unique to this disease. These include the formation of microsclerotia and setae within lesions and lesions on the stem that are sunken into the plant tissue. The following details the key features.

3.9 Biology

3.9.1 Identification

Morphological Description:

Colonies are dark brown to black, with a felt of greyish mycelium through which the tufted to irregular, often confluent, black sclerotia protrude, occasionally with scattered tufts of pale grey to white mycelium. Conidial masses are honey coloured. Sclerotia are abundant. Conidia falcate, apices obtuse, guttulate, 15.5 – 24.0 X 3.5 – 4.0 μm (refer figure 28). Appressoria abundant, clavate or circular, sometimes irregular, 11 – 16 X 8 – 9.5 μm, often becoming complex (Sutton 1980).

3.9.2 Symptoms

Leaf lesions and premature leaf drop:

In most lentil crops, the first symptoms of anthracnose appear before flowering, when the plants have 8 to 12 nodes on the main stem. This is also the time when the first tendrils form, and approximately a week before flowers start to open. If there is a large amount of inoculum in the field the first

symptoms may appear earlier. The initial symptoms of lentil anthracnose are greenish water-soaked lesions on the lower stems and leaves that become necrotic with time. Tan coloured lesions of variable size develop on the lower leaflets and the most severely affected leaflets die prematurely and drop to the ground. Creamy white lesions are also sometimes evident on the upper foliage (see figures 8 - 11).

Stem lesions:

Lesions on stems develop soon after the appearance of leaf lesions, during flowering, primarily at the base of the plant. Stem lesions may be small, brownish with a black border, or larger, stretching along the stem. As the season progresses, more and more golden-brown lesions develop at the stem base, as well as on the upper part of the stems, and many stems are girdled. Finally there is a marked blackening of old infected tissues due to the production of stromatic mycelium (microsclerotia) under acervuli (see figures 13 - 19).

Microsclerotia (survival structures):

Small, pinhead-sized fungal structures (microsclerotia) form on the older infected plant tissue. They may be seen with the unaided eye in the centre of stem lesions or more easily with a hand lens (10 -15 x magnification). Each microsclerotia consists of a few hundred cells with thick, black cell walls that protect the fungus from colonisation by other micro-organisms. Microsclerotia enable the fungus to survive between lentil crops either on the plant debris or free in the soil. They remain viable longer when buried in the soil by tillage than when left exposed to weather extremes on the soil surface. These fungal structures survive on dead lentil debris or in the soil during periods when a host is not available (see figures 13, 15 & 19).

Wilt:

Anthracnose causes defoliation and stem girdling, which inhibits utilisation of water and nutrients. The fungus penetrates the vascular tissue and girdles the stem resulting in wilting of the entire plant. As a result, large areas of brown and dying plants can be found in the field (see figures 25 - 27).

3.9.3 Disease cycle

C. truncatum can survive in several forms, including infected seed, as microsclerotia and on infested trash. Seed infection levels of 2-3% have been reported from severely diseased crops (Morrall, 1997), but generally levels of 0.5 – 1% are more common. Transmission of the disease from seed to seedling has not been demonstrated in the glasshouse, but anecdotal evidence from Canada suggests that transmission of the disease from seed has occurred in the field (L. Buchwaldt and R. Morrall pers. comm.).

The pathogen can survive as microsclerotia on crop residues for up to four years under Canadian conditions. Infested trash, which includes pod walls and small stem fragments, can carry microsclerotia, which can initiate infection in subsequent lentil crops. When the plant debris decomposes the microsclerotia can survive free in the soil. Canadian research found that survival of *C. truncatum* microsclerotia increased from 12 months, when left on the soil surface, to 4 years when buried on lentil debris (Buchwaldt *et al.* 1996).

Spores are initially dispersed from old lentil debris near the soil surface to newly establishing lentil plants by rain splash. Symptoms usually appear 7 – 8 days following infection. Newly infected plants produce new generations of spores that disperse to infect neighbouring plants by rain splash. Spores are produced in large numbers on infected host material, even leaflets that have dropped onto the ground. Observations in Canada have revealed that the number of rainfall events is more important than the total amount of rainfall. Once the pathogen has entered the plant the fungus will grow regardless of temperature and humidity. Chongo and Bernier (2000b) found that the optimal conditions for lentil anthracnose development were temperatures of 16 – 24°C in conjunction with 18 – 24 hours of leaf wetness.

Although many plant species can be infected with *C. truncatum,* including many pulse crop and weed species, there is considerable evidence to suggest that there are host specific pathotypes. Some strains of *C. truncatum* have been investigated for use as mycoherbicides because of their very narrow host range (Boyette 1991). Host range testing in Canada has shown faba bean (*Vicia faba*), (refer figure 24), and vetch (*Vicia sativa*) to be highly susceptible when inoculated with isolates of *C. truncatum* from lentil (Buchwaldt *et al*. 1996). Under greenhouse testing, field pea (*Pisum sativum*) has also become infected, but with minor symptoms (Morrall *et al.* 1989). In Canadian glasshouse trials, Gibson (unpublished data) found lentil, faba bean, field pea, grass pea (*Lathyrus sativus*), tangier pea (*Lathyrus tingitanus*) and yellow vetch (*Vicia sativa*) to be susceptible to *C. truncatum*, isolated from lentil. Weidemann *et. al*. (1988) found that isolates of *C. truncatum*, isolated from *Pisum sativum*, when inoculated onto 21 other leguminous species only caused major damage on *P. sativum*, *Vicia ervillia* and *Lathryrus odoratus. Lens culinaris* developed only minor symptoms, even at high conidial concentrations, suggesting it would not become infected under normal field conditions.

Field pea crops have been considered to play a role in the carry over of lentil anthracnose inoculum at low levels when rotated with lentil under field conditions in Canada (Morrall 1997). In addition, *C. truncatum* has also been isolated from faba bean seed in Hungary (Simay 1990), suggesting that faba bean seed may also potentially assist in the survival and spread of inoculum between seasons.

Most recently, Buchwaldt *et al.* (2004) found the existence of two groups of *C. truncatum* isolates from lentil in western Canada, with different virulence on lentil differentials. These two races have been designated Ct1 and Ct0. Resistance to Ct1 has been identified in a number of lentil accessions (Buchwaldt *et al.* 2004), and resistance to Ct0 has been identified in a single accession sourced from Russia (Buchwaldt and Diederichsen 2004). The two races have been isolated with similar frequency from lentil plants in commercial fields. Race Ct0, to which limited resistance has been identified, presents a high risk to lentil production (Buchwaldt *et al.* 2004).

3.9.4 Dispersal

Dispersal of *C. truncatum* can occur in several forms, these being infested debris, dust and seed. Each can be important in the spread and establishment of the disease:

- Under Canadian conditions significant spread of the pathogen occurs during harvest operations. Small fragments of pod wall and stem pieces carrying microsclerotia can be blown into surrounding paddocks following harvest and allow the pathogen to move considerable distances away from the infested crop (refer figure 23). These same small fragments can be carried within infested seedlots, in other bulk commodities and in machinery over larger distances.
- The pathogen also has potential to be transmitted as infected seed. While seed to seedling transmission has not been reported, this pathway of dispersal cannot be discounted.

Spores of *C. truncatum* can be dispersed before the crop reaches maturity, generally over shorter distances.

- Spores are generally dispersed within crops by rainsplash. This will spread the disease from primary sources of infection within the crop to surrounding plants. Windblown rain may create aerosols, which may spread infection between neighbouring crops.
- Spores may adhere to clothing, machinery or animals, which may come in contact with sporulating lesions and spread to nearby crops.

3.10 Assessment of likelihood

The following risk analysis is based on the methodology in Biosecurity Australia's guidelines on Import Risk Analysis (2001).

3.10.1 Entry potential

Entry potential is **Low**, but possible given the following factors

- Australia currently imports 2000 – 3000 tonnes of whole green lentils for human consumption from Canada annually. Current AQIS import conditions require that imported consignments be

grown in an area free of *C. truncatum* and be accompanied by a phytosanitary certificate. Despite this legislation, there is no guarantee that the pathogen cannot enter via infected seed or infested lentil trash bearing microsclerotia that may accompany the consignment.

- In addition, the pathogen is also seedborne in a number of other legume species (see Section 3.7.1) Seed of these species entering Australia may also carry the pathogen and hence pose a possible pathway of entry.
- There is a high frequency of travel between Canada where the pathogen exists and Australia, increasing the possibility of viable spores or microsclerotia adhering to footwear and clothing, and coming into contact with a host plant.

3.10.2 Host range potential

Host range potential is **Medium** as *C. truncatum* has a moderately complex host range involving one or more plant families.

3.10.3 Establishment potential

Establishment potential is considered to be **High**, as

- Races of *C. truncatum* already occur in Australia on other crop host species demonstrating that suitable conditions do occur in Australia for the pathogen to survive. In addition, host species such as vetch, faba bean and field pea are grown in close rotation with lentil, allowing the pathogen to survive and spread, even in the absence of lentil hosts.
- Climatic conditions between regions in Canada where the disease already occurs and areas of Australia are similar.
- Microsclerotia of the pathogen can survive in the soil for up to 4 years in the absence of a host plant.
- Current commercial lentil cultivars in Australia are highly susceptible to lentil anthracnose.

3.10.4 Spread potential

Spread potential is considered to be **Medium**, given the following

- Spores are splash dispersed, rain splash can carry spores short distance to surrounding plants. Wind blown rain can carry spores longer distances into neighbouring crops.
- The host range of the pathogen includes lentil, faba bean, vetch and grass pea. Field pea can be infected by the pathogen but develop minor symptoms.
- Microsclerotia of the pathogen can be transported over large distances in infested grain and harvesting equipment into new areas.

- Wind blown plant debris could spread the pathogen over moderate distances following harvest into adjacent paddocks.

3.11 Overall entry, host range, establishment and spread potential

Low - The probability of entry, establishment and spread is determined by combining the likelihoods of entry, host range, establishment and spread.

3.12 Assessment of consequences

3.12.1 Economic impact

Extreme – This disease has the potential to greatly downsize the lentil industry in Australia in a similar manner to the ascochyta blight outbreak in chickpea in 1998. An outbreak of lentil anthracnose would result in a dramatic reduction in the area of production, due to increased costs of production making lentils less competitive compared to other crops. Faba bean production may also be affected as this crop species can be infected by the lentil anthracnose pathogen.

A substantial loss would also be incurred in the year of the outbreak. This not only includes lost production but also indirect impact on other business sectors such as other agricultural enterprises, storage, transport, manufacturing and wholesale trade. The losses would be similar to those incurred as a result of the outbreak of ascochyta blight in chickpeas in 1998 which has been calculated to have cost the Wimmera region in Victoria, $62 million.

Under Canadian conditions, lentil anthracnose causes yield losses of up to 60% if left uncontrolled.

3.12.2 Environmental impact

Negligible - There is no potential to degrade the environment or otherwise alter the ecosystem by affecting species composition or reducing the longevity or competitiveness of wild hosts.

3.12.3 Social impact

Moderate - The reduction in the value of production would be expected to cause moderate social impact with significant losses to the local and broader community.

3.13 Combination of likelihood and consequences to assess risks

- Economic risk – **Extreme** – specific action is immediately required to reduce risk.
- Environmental risk – **Low** – manage through routine procedures.
- Social risk – **High** - adoption of generic risk treatment plans will reduce the risk to suitable levels.

3.14 Surveillance

Maintaining vigilance for exotic pathogens within the grains industry is clearly important when recent incursions of lupin anthracnose and chickpea ascochyta blight in Australia may have been avoided had industry been aware of exotic grains threats and their identification. From experiences in Canada, lentil anthracnose had most likely been present there for some time before being identified (Morrall 1997). Lentil growers and agronomists should be encouraged to report any unusual symptoms to their State Departments of Agriculture where an experienced plant pathologist can perform identification. If effective control and possible containment of an exotic plant disease is to occur then rapid identification of the disease is necessary.

3.15 Diagnostics

Diagnosis of lentil anthracnose is a two-stage process. Firstly, a preliminary microscopic examination is undertaken to determine whether disease symptoms and pathogen morphology are consistent with lentil anthracnose or an endemic disease, such as ascochyta blight or botrytis grey mould. The primary diagnostic test, a PCR test is undertaken to determine whether the pathogen is the lentil-attacking strain of *Colletotrichum truncatum*. An experienced plant pathologist should perform the preliminary examination. The primary test requires sample processing in a specialised laboratory capable of molecular techniques. A secondary test for *C. truncatum* is a detailed morphological examination of fungal cultures. This would require the skills of an experienced mycologist.

The diagnosis of lentil anthracnose would need to be performed quickly and accurately. The accompanying report describes methods of sampling and diagnosing lentil anthracnose using conventional PCR methods.

3.16 Training

Due to the similarity of lentil anthracnose symptoms to those of other diseases affecting lentil, such as ascochyta blight, growers and field agronomists need to be aware of the importance of this disease. While it is not possible for all personnel in the field to be trained in disease symptoms and identification, a general awareness of crop disorders and an awareness of local diagnosticians are critical for early disease detection and hence industry protection.

4.0 Diagnostic protocol

4.1 The diagnostic test/s and diagnostic sequence

The primary diagnostic test for the detection of *Colletotrichum truncatum* from lentil is a PCR-based assay, using highly specific diagnostic molecular markers. This test is able to distinguish between *C. truncatum* isolates from lentil and isolates of *C. truncatum* from other host plants, and other pathogens also found on lentil (such as *Ascochyta lentis* and *Botrytis* spp.). This test can use either fungal cultures or infected plant material as the source of DNA. Currently this is the only PCR diagnostic test available worldwide that is capable of distinguishing between isolates of *C. truncatum* from lentil and other host plants. The secondary test for *C. truncatum* is a detailed identification based upon morphological features, but this method cannot distinguish *C. truncatum* isolates from different host plant species.

4.2 The initial samples

It is highly likely that samples received to the laboratory for diagnosis will have been dispatched with the sender unaware that the sample may be an exotic pathogen. This being the case, it is important that once formal diagnosis has been made and confirmed as a possible exotic incursion that the diagnostic procedures and line of responsibilities are clearly understood. If a sample is received that is potentially exotic, it is the responsibility of the diagnostician to consult and notify the relevant State Authorities and pathology experts.

4.2.1 Documentation

Accurate documentation of specimens, sub-sampling and sample processing is vital if large numbers of samples are to be inspected and processed. Trace back of specimens needs to be an accurate, easy task, especially if results are to be released to the public.

- It is vitally important that all possible details are collected regarding the origin of samples. Information collected here will give some indication of the source of disease, and the likelihood of spread. The information will also form the starting point of any crop surveys. Any results and findings should also be kept with the sample log sheets.

Specimen worksheets will also be necessary for recording diagnostic processing and results.
The Appendix at the back of this manual contains worksheets for sampling of crops (Appendix 1), trace back of seedlots (Appendix 2) and laboratory worksheets (Appendix 3).

4.2.2 Sample storage

- Fresh plant samples should be stored under cool conditions either in a coolroom or refrigerator. It is important that samples not be allowed to „sweat" in sealed plastic bags, this allows for rapid deterioration and contamination of the sample and which may render it non-viable for diagnostic purposes.
- Wrapping fresh plant samples in damp (not wet) paper and storing in open containers can be a good method of maintaining plant samples for short periods. If possible, sub samples of infected plant material should be stored in a –80ºC freezer for DNA preservation and clearly labelled.

Ideally a sub culture should be taken from the fresh plant sample as soon as possible after receival. Fungal cultures can then be placed into long term storage for future reference.

4.2.3 Sample handling and subsampling

If suspect samples are received:

- Pictures should be taken, if possible, of the intact sample showing representative disease symptoms. These may be kept with sample records.
- The pathogen should be subcultured from infected tissue as soon as possible. This allows for diagnosis to proceed. Agar plates should be clearly labelled so that plate cultures can be traced back to original plant specimens. Sub samples of the plate culture may be forwarded to other laboratories for diagnosis or placed into longer-term storage or future reference.
- In addition to plate cultures, sub samples of fresh plant specimens may also be forwarded to other laboratories for diagnosis.
- All diagnostic details have to be recorded so that trace back can occur for specimens. This includes plate cultures as well. This becomes vitally important if growers have to be notified of positive identifications.

4.2.4 Visual symptoms

Coloured plates of symptoms are supplied in Section 8.

4.3 Further samples

Once initial samples have been received and preliminary diagnosis made, follow up samples to confirm identification of the pathogen will be necessary. This will involve sampling directly from the infected crop, and sampling crops over a larger area to determine the extent of disease distribution. The total number of samples collected at this point may run into the hundreds or even thousands. It is vital that a system of sample identification is determined early in the procedure to allow for rapid sample processing and accurate recording of results. Follow up samples will be forwarded to the nominated diagnostic laboratories for processing.

4.3.1 Sample collection, transport and storage

Sample collection.

Samples should be initially collected over a representative area of the infected crop to determine the disease distribution. The disease will appear as patches within the crop given the nature of dispersal of the pathogen. Depending on the stage of infection the symptoms may appear as:

- Plants with premature defoliation
- Plants with stem lesions
- Patches of plants dying within the crop

It is important to note the distribution of disease in the initial crop, as this will indicate whether the disease has been seed-borne, carried on trash from adjacent paddocks or originated from contaminated machinery or human movement.

It is vitally important that all personnel involved in crop sampling and inspections take all precautions to minimise the risk of disease spread between crops by decontaminating between paddocks. Appendices 4 and 5 list a number of procedures to follow when decontaminating during crop inspections.

Sampling of crops over a district area and further will be based upon the origins of the initial suspect sample.

Factors to consider will be:

1. The source of seed of the infected crop and how long that seed has been used by the grower (see Appendix 2).
2. If any other lentil crops have been sown from the same source seed.
3. The proximity of other pulse crops, especially lentils, to the initial infected crop, both in the current growing season and previous season. Faba bean and vetch crops should also be considered as these crops can also host the pathogen. This will include the growers own pulse crops and pulse crops on neighbouring properties.
4. What machinery movements have been made into the infected crop.
5. The extent of human movements into the infected crop. A possible link to recent overseas travel should also be considered.

All foliage can become infected by *C. truncatum*, this includes leaves, stems and pods. Samples should be collected that represent a range of symptoms observed in the infected crop. Preferably enough material should be collected to allow for immediate processing and retention of a portion that can be placed into long term storage as a reference.

Sample transport

Samples should be treated in a manner that allows it to arrive at the laboratory in a fresh, well-preserved state. An esky with ice packs or portable fridge should be carried when sampling crops. Samples should be wrapped in damp newspaper, bundled into a plastic bag and clearly labelled.

Sample storage

Samples should be processed as quickly as possible after sampling from the field if sub cultures are to be made from infected tissue. Once removed from the field, fresh plant samples can deteriorate and become contaminated by other mould fungi and bacteria, which may prevent successful sub culturing of the pathogen. Sub culturing should be done within three to four days after sampling from the field. Infected plant tissue to be used for PCR analysis can be placed in a -80^0C freezer and stored for an indefinite period under these conditions without damaging fungal DNA.

Long term storage of specimens can occur as fungal cultures that can be freeze dried for future reference (without loss of viability) or as deep frozen plant specimens maintained at -80^0C, which can be used to extract DNA.

4.3.2 Sample locations

It is important to record the precise location of all samples collected, preferably using GPS, or if this is not available, map references including longitude and latitude and road names should be recorded. Property names and owners should also be included where possible.

4.4 Confirmation of diagnosis

It is important that all diagnoses of suspected exotic and emergency pathogens are undertaken according to the following parameters: the lab/diagnostician has expertise in this form of diagnosis, the test is undertaken as described in this manual, the results are confirmed by diagnosis in another recognised laboratory or another diagnostician and where possible diagnosis is confirmed by a second method.

5.0 Preliminary examination

5.1 Introduction

Morphological characters, while not being diagnostic to the level of host specific strain, can confirm the presence of *Colletotrichum truncatum* on lentil.

5.2 Method

5.2.1 Preliminary examination

1. The infected plant material is examined for the presence of microsclerotia or other fruiting bodies under the dissecting microscope. If fruiting bodies are present their identity should be determined under the compound microscope (ie, microsclerotia or pycnidia). If fruiting bodies are found to be pycnidia, the causal pathogen is most likely *Ascochyta lentis*.
2. To be certain of the identity of the pathogen infected plant tissue should be plated onto agar media (refer section 7).

5.2.2 How to distinguish anthracnose and ascochyta symptoms.

Anthracnose and ascochyta blight of lentil, caused by *A. lentis*, cause similar symptoms on leaves and pods, but are slightly different on stems. Stem symptoms are notably different, since anthracnose penetrates deeper and creates dents in the stem surface, while ascochyta lesions are more superficial. To determine which disease is present, it is helpful to look for lesions where the pathogen has produced pinhead-sized, black structures. In an ascochyta lesion, these structures (pycnidia) are almost round, rarely touching one another, and partially embedded in the plant tissue (see figure 20). If anthracnose is present, the structures (microsclerotia) are irregularly shaped and some are clumped together (see figures 13, 15 & 19). Most characteristic of anthracnose, however, are the tiny black hairs (setae) sticking up from the lesion, which can be seen with the naked eye or a hand lens at 10 – 15 magnification. Setae will grow out of the lesion even if microsclerotia are not present (see figure 13). Microsclerotia of anthracnose are easiest to observe in stem lesions, and pycnidia of ascochyta are often found in leaf lesions (Buchwaldt 2005).

5.2.3 Encouraging setae development.

The appearance of setae within a suspect lesion is a characteristic feature of lentil infected by *C. truncatum*. Often setae can be encouraged to develop within 24 hours if infected plant pieces are placed within moist chambers. Microsclerotia, while also characteristic of the disease, will take several more days to develop.

Portions of plants with lesions can be incubated within a moist chamber to induce the formation of setae. Infected plant parts can include both leaf and stem lesions. Microsclerotia will also form under these conditions but will take several more days. Setae can develop overnight under ideal conditions,

but usually take 24 – 36 hours. The easiest moist chambers to prepare are made by placing moist filter paper, dampened with distilled water, in the bottom of a petri dish and placing the specimen on the moist paper. These chambers allow the specimen to be sealed within the plate and can be viewed under a dissecting microscope without disturbing the specimen.

Normally moist chambers are incubated at room temperature with diurnal lighting. Specimens should not require surface sterilising as this may inhibit fungal growth. However, any plant tissue covered with soil should be washed in gently running tap water prior to setting up in a moist chamber.

6.0 Identification of pathogen (primary diagnostic tests)

6.1 Introduction

Two Primary tests are available for the detection of *Colletotrichum truncatum* using real-time PCR and traditional PCR methods. Following preliminary examination of the suspect plant sample, analysis of the sample to determine if the causal pathogen is *C. truncatum* will be required. The tests detailed below will distinguish *C. truncatum* from other common lentil-attacking foliar pathogens and also identify the lentil-attacking strain of *C. truncatum* from other *C. truncatum* strains already in Australia that are present on other leguminous hosts. Refer to Appendix 6 for a separate listing of all reagents and solutions required, including suppliers and catalogue numbers.

6.1.1 DNA Extraction

6.1.1.1 General Items required

1. Samples - plate culture or infected/suspect plant tissue
2. 2-20 μL pipettes, 20-200 μL pipettes, 200-1000 μL pipettes, and sterile tips
3. Balance (that weighs to at least two decimal places) and weighboats
4. Disposable gloves and paper towels
5. Microcentrifuge
6. Sterile microcentrifuge tubes
7. Vortex shaker
8. Sharps container
9. Sterile scalpel blades
10. Screw cap tubes and lids (Axygen Catalogue No. SCT-200-SS-C)
11. Waterbath or heatblock set at 55°C
12. Fume hood
13. Autoclaved mortar and pestle
14. Liquid nitrogen
15. Sterile distilled water

Solutions/ Reagents required

6.1.1.2 - Extraction buffer (pH 5.5) (refer Ford *et al.,* 2004).

	1L	final
Cetyltrimethyl ammonium bromide (CTAB)	20.0 g	2% w/v
NaCl	81.82 g	1.4 M
0.5M EDTA (pH 8.0)	40 mL	20 mM
1.0M Tris HCl (pH8.0)	100 mL	100 mM

- Dissolve solids and mix solutions in 1L sterile RO water.
- Autoclave before use.
- Store at room temperature.

NB. EDTA is a hazardous substance. Read MSDS before use

6.1.1.3 - 1.0M Tris HCl (pH8.0)

- 12.1g Tris base dissolved in 100 ml sterile RO water
- pH to 8.0 with concentrated HCl
- autoclave before use
- store at room temperature

6.1.1.4 - 0.5M EDTA (pH8.0) ethylenediamine trisodium acetate

- 18.6g EDTA dissolved in 100ml sterile RO water
- pH to 8.0 with NaOH while stirring. Solid will not dissolve until close to pH8.0
- autoclave before use
- store at room temperature

6.1.1.5 - Phenol:chloroform:isoamyl alcohol. (25:24:1).

- Buy solution already mixed in these ratios
- Store at 4^{o}C

NB. Phenol is a carcinogen and appropriate gloves, safety glasses and laboratory coats must be worn at all times. Phenol must always be used in a fume hood.

6.1.1.6 - TE Buffer

	1 L	final
1M Tris HCl (pH 8.0)	10 mL	10 mM
0.5M EDTA (pH 8.0)	2 mL	1 mM

- Mix reagents in 1L sterile RO water
- Autoclave before use
- Store at room temperature

6.1.1.7 - 10mg/ml RNAse A

	10 mL	final
RnaseA	100 mg	10 mg/mL
1M Tris-HCl pH7.5	100 μL	10 mM
5M NaCl	3 μL	15 mM

- Dissolve components in 10 mL sterile RO water
- Boil for 15 min
- Cool slowly to room temperature
- Aliquot into 1mL volumes
- Store at –20^{o}C

6.1.2 DNA collection from suspect lentil plant tissue

The extraction steps used to obtain DNA from samples are done at room temperature.

1. Take infected or suspect leaf and stem tissue (100-400ng) and use directly or cut out lesions to use specifically for DNA extraction
2. Add small amount of liquid nitrogen and grind to a fine powder
3. Once melted but still cold, add 1.5 mL of CTAB buffer pH 5.5 and grind further
4. Carefully pour CTAB suspension into a fresh 1.5 mL microcentrifuge tube
5. Vortex suspension well
6. Incubate samples @ 55^0C for 20-30 mins
7. Spin suspension at 13,000 rpm for 5 mins in bench top centrifuge
8. Remove 500 µL of supernatant to fresh tube (store remainder at @ 20^0C for further DNA extraction if required)
9. Add 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1). Mix by gentle inversion and spin as above.
10. Remove supernatant (top layer) carefully to fresh tube, be careful not to take any of the interface.
11. Add 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) to supernatant
12. Repeat mixing and spinning
13. Remove supernatant to fresh tube (should be clear - if not try more extraction steps)
14. Add 2.5 volumes of ice cold 100% Ethanol
15. Mix by inversion and leave to precipitate nucleic acid at -20°C for 30 mins
16. Spin at 13,000 rpm for 10 mins
17. Carefully pour off and discard 100% ethanol
18. Add 200 µL of 70% ethanol, invert tube several times and spin at 13, 000rpm for 5 min.
19. Carefully pour off 70% ethanol and blot excess on sterile tissue paper
20. Dry pellet under vacuum (pellet should be white or transparent, not coloured)
21. Resuspend pellet in 50 µl TE buffer
22. Add 2µl of 5units/µl RNAse A and leave on bench at RT for 5 mins
23. Store total genomic DNA @ -20^0C.

6.2 Detection of the Pathogen using traditional PCR

6.2.1 General Items required

1. 0-2 μL, 2-20 μL, 20-200 μL, and 200-1000 μL pipettes and sterile tips
2. 0.2 mL thin walled sterile PCR tubes
3. Centrifuge
4. Disposable gloves
5. Freezer, ice
6. Gel electrophoresis tanks, rigs and racks
7. DNA Molecular Weight markers
8. Sterile microcentrifuge tubes
9. Microwave
10. Power pack
11. QPCR machine (Strategene MX3000P) or conventional PCR machine
12. UV transilluminator with digital capture equipment

Solutions/Reagents required

6.2.1.1 - Traditional PCR Primers and expected product sizes

SCARGO2 primers were designed from a RAPD band
SCARG02 forward: 5‟-GGC ACT GAG GAC GTC CTG AC-3‟
SCARG02 reverse: 5‟-GGC ACT GAG GTA AGC GCT TAG G-3‟
Expected product size: 1600bp
The SCARG02 primers only amplify a product from the lentil *C. truncatum* genome

The SCARG15 primers were designed from a RAPD band
SCARG15 forward: 5‟-ACT GGG ACT CGC TTT TCT CAG TCC-3‟
SCARG15 reverse: 5‟-ACT GGG ACT CAA CAA AAG AGC G-3‟
Expected product size: 900bp
The SCARG15 primers only amplify a product from the lentil *C. truncatum* genome

Both of the SCARG02 and SCARG15 diagnostic PCR products were too large for adaptation to a REAL TIME PCR system.

6.2.1.2 - PCR controls

Positive control 1: DNA extract from lentil *C. truncatum* isolate culture

Positive control 2: DNA extract from lentil tissue infected with *C. truncatum* using the method described above

Negative control 1: Uninfected plant control = DNA extract from uninfected plant tissue of the same species as that used for the positive control

Negative control 2: DNA extract from a non lentil *C. truncatum* isolate, i.e. another *Colletotrichum* species, another common lentil fungal foliar pathogen such as *Ascochyta lentis*, and/or *C. truncatum* from another host species

Negative control 3: A non-template control, ie. an aliquot of the PCR Master Mix minus DNA template

6.2.1.3 - PCR reagents

- Taq polymerase reagent kit
  - 10x PCR buffer
  - Taq polymerase (5units/µl)
  - 50mM MgCl
  - Mix of 20mM each dNTPs

6.2.1.4 - 5 x TBE Buffer

	1 L	final
Tris base	54.0 g	0.4 M
Boric acid	27.5 g	0.05 M
0.5M EDTA pH 8.0	20.0 mL	0.001 M

- Dissolve components in 1 L sterile RO water
- Store at room temperature.

6.2.1.5 - 1.4% Agarose with ethidium bromide

	100 mL	Concentration
DNA grade agarose	1.4 g	1.4%
1 x TBE	100 mL	1x
10mg/ml Ethidium bromide	3 μL	0.3 mg/mL

- Dissolve agarose in TBE by heating in microwave for 1 min 20 sec
- Mix by shaking
- Cool to hand temperature and add Ethidium Bromide, mix
- Pour into get tray with comb to set

NB. Phenol is a carcinogen and appropriate gloves, safety glasses and laboratory coats must be worn at all times. Phenol must always be used in a fume hood.

6.2.1.6 - 6x Loading dye

	50 mL
1 x TE	5 mL
Glycerol	25 mL
bromophenol blue	0.125
xylene cyanoll FF*	0.125

- Make up in 50 ml RO water
- Store at room temperature

*__NB__. xylene cyanoll FF is an irritant (in powder form)

6.2.2 Traditional PCR analysis

This method may be used with the SCARG02, SCARG15 and CT primer sets

1. Label sterile PCR tubes
2. Prepare "Master Mix" on ice in a sterile microcentrifuge tube

   The standard 25 µl PCR reaction contains:

   2.5 µl 10 x PCR buffer (minus $MgCl_2$)

   1.8 µl $MgCl_2$ (50 mM)

   0.3 µl dNTPs (20 mM)

   0.2 µl Taq DNA polymerase (5 units/µl)

   1.0 µl Forward primer (10 µM)

   1.0 µl Reverse primer (10 µM)

   13.2 µl H_2O

   20 µl + 5 µl of template DNA per PCR reaction (or an additional 5 µl of H_2O for the non-template control)

   Total volume = 25 µl per PCR reaction

   To prepare the "Master Mix", multiply the above volumes by the number of samples (including controls) to be tested. Add 20 µL of Master Mix to each PCR tube.

3. Add 5µl of each template (total DNA extract or non template H_2O control) to each corresponding PCR tube.
4. Subject PCR reactions to the following thermocycle conditions:

   3 min @ 94^0C x 1 cycle

   30 sec @ 94^0C
   30 sec @ 60^0C } x 35 cycles
   1 min @ 72^0C

   5 min @ 72^0C x 1 cycle

   Hold at 15^0C
5. Mix 10 µl of each PCR sample with 2 µl of a 6 x agarose gel loading/tracking dye (Invitrogen, USA)
6. Load samples onto a 1 x TBE buffer 1.4 % agarose gel
7. Run gels in 1 x TBE buffer at 80V for 90 minutes or until the bromophenol blue dye front has migrated to within a centimeter from the end of the gel
8. Stain the gel in a 50ng/µl fresh solution of ethidium bromide for 20 minutes
9. Visualise and photograph the gel under UV-light

6.2.3 Traditional PCR results

The following figure shows the expected amplification products after PCR with the SCARG02, SCARG15 and CT primer sets from *C. truncatum* infected lentil, and their respective sizes. This also demonstrates that these primers may be multiplexed to increase the validity of a positive PCR result.

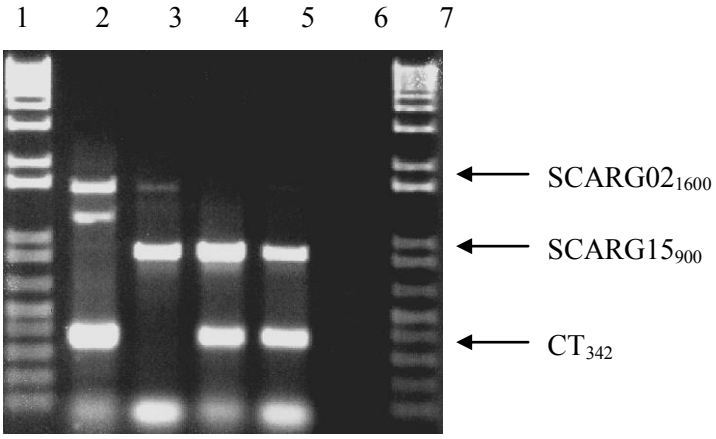

Figure 4. The expected amplification products after PCR with the SCARG02, SCARG15 and CT primer sets from *C. truncatum* infected lentil.

Amplification products generated from naturally infected plant tissue with multiplexed primer combinations. Where Lanes 1, 7 = 1kb mw ladder (Invitrogen, USA); Lane 2 = amplification using SCARG02 and CT primer sets; Lane 3 = amplification using SCARG02 and SCARG15 primer sets; Lane 4 = amplification using SCARG15 and CT primer sets; Lane 5 = amplification using SACRG02, SCARG15 and CT primer sets; Lane 6 = non-template control.

These products may be detected on an ethidum bromide stained gel at the following sensitivities:
SCARG02$_{1600}$: 1 pg of purified fungal DNA (able to detect the pathogen in 0.1 ng of diseased lentil tissue)
SCARG15$_{900}$: 10 pg of purified fungal DNA
CT$_{342}$: 10 pg of purified fungal DNA

6.3 Detection of the pathogen using real-time PCR

6.3.1 General Items required

13. 0-2 μL, 2-20 μL, 20-200 μL, and 200-1000 μL pipettes and sterile tips
14. 0.2 mL thin walled sterile PCR tubes
15. Centrifuge
16. Disposable gloves
17. Freezer, ice
18. Gel electrophoresis tanks, rigs and racks
19. DNA Molecular Weight markers
20. Sterile microcentrifuge tubes
21. Microwave
22. Power pack
23. QPCR machine (Strategene MX3000P) or conventional PCR machine
24. UV transilluminator with digital capture equipment

Solutions/ Reagents required

6.3.1.1 - Real-time PCR Primers and expected product size

The CT primers were designed from the internal transcribed spacer (ITS) regions of the 18-25S ribosomal (r) DNA sequence

CTFP2 forward: 5‟-TACCGTAGGGCCCCAACACCAAGCC-3‟

CTRP1 reverse: 5‟-CAACTGTTGCTTCGCCGGGCAGGAGGACG-3‟

Expected product size: 342 bp from lentil *C. truncatum*

338 bp from soybean *C. truncatum**

*Digestion at a restriction enzyme site unique for the lentil isolate, BsiEI, produced a 285 bp fragment, readily discriminating it from the soybean isolate.

6.3.1.2 - PCR controls

Positive control 1: DNA extract from lentil *C. truncatum* isolate culture

Positive control 2: DNA extract from lentil tissue infected with *C. truncatum* using the method described above

Negative control 1: Uninfected plant control = DNA extract from uninfected plant tissue of the same species as that used for the positive control

Negative control 2: DNA extract from a non lentil *C. truncatum* isolate, i.e. another *Colletotrichum* species, another common lentil fungal foliar pathogen such as *Ascochyta lentis*, and/or *C. truncatum* from another host species

Negative control 3: A non-template control, ie. an aliquot of the PCR Master Mix minus DNA template

6.3.1.3 – Master Mix

Brilliant® SYBR® green QPCR master mix (Stratagene #600548)

6.3.2 Real-time PCR analysis

This method may be used with the CT primer set only

1. Label sterile PCR tubes on the lids only so ink does not interfere with acquisition of SYBR green data.
2. Prepare PCR reactions on ice as follows:

   The standard 25 µl PCR reaction contains

   1 x Brilliant® SYBR® green QPCR master mix (Stratagene #600548)

   0.2µM of each primer

   5-25ng template DNA

   sterile H_2O to a final volume of 25 µl

3. Subject PCR reactions to the following thermocycle conditions:

   10 min @ 95^0C x 1 cycle

   30 sec @ 95^0C
   1 min @ 62^0C x 35 cycles
   90 sec @ 72^0C

   3 min @ 72^0C x 1 cycle

   Hold at 15^0C

Check Real Time amplification products on a 1.4% agarose gel as previously described for traditional PCR

Set the detection thresholds on the QRT-PCR machine (Strategene MX3000P) as follows (to exclude any potential false positives):

Fluorescence = 20000 dR (Y-axis)

Cycle length = 30 cycles (X-axis)

Note – as little as 0.5pg of purified fungal DNA can be detected with 36 cycles

6.3.3 Real-time PCR results

The following figure shows the amplification of the expected 342 bp product amplified from 0.5 pg – 500 pg of lentil *C. truncatum* DNA, after PCR with the CT primer set and the Sybr Green® PCR mix.

1 2 3 4 5

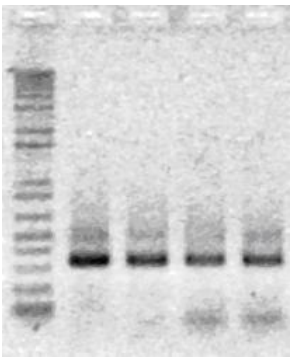

Figure 5. The expected 342 bp product amplified from 0.5 pg – 500 pg of lentil *C. truncatum* DNA, after PCR with the CT primer set and the Sybr Green® PCR mix.

Amplification products generated from purified fungal DNA. Where Lanes 1 = 1kb mw ladder (Invitrogen, USA); Lane 2 - 5 = amplification using the CT primer set and lentil *C. truncatum* isolates 99-70946, 99-71646, 99-72126 and 99-70461.

The following is the general QRT-PCR profile for the CT_{342} diagnostic marker amplified from 500pg of purified lentil *C. truncatum* DNA

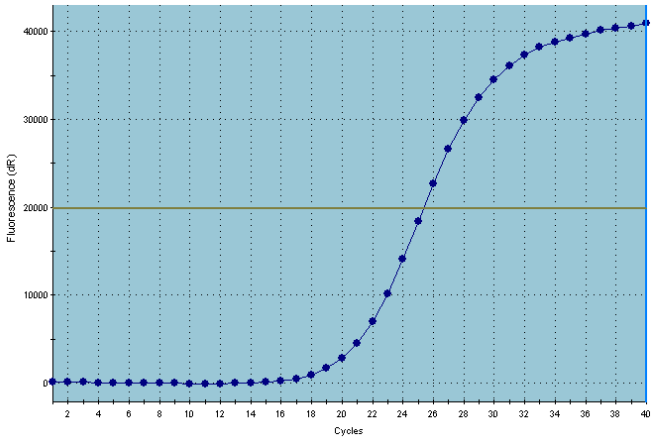

Figure 6. The general QRT-PCR profile for the CT_{342} diagnostic marker amplified from 500pg of purified lentil *C. truncatum* DNA

The following is the QRT-PCR profile for the CT_{342} diagnostic marker when using 0.5 pg of purified lentil *C. truncatum* DNA. The fluorescence threshold is set at 20000 dR (Y-axis) and 30 cycles (X-axis) are used, to exclude any potential false amplifications

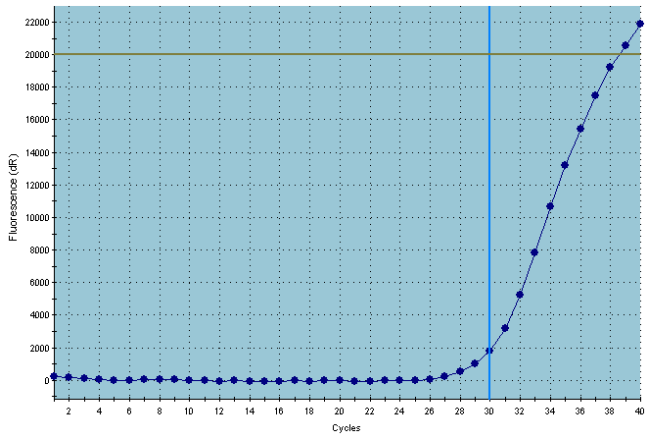

Figure 7. The QRT-PCR profile for the CT_{342} diagnostic marker when using 0.5 pg of purified lentil *C. truncatum* DNA.

7.0 Confirmation of Diagnosis

7.1 Morphological Inspection of *Colletotrichum truncatum.*

7.1.1 Introduction

Morphological characters, while not being diagnostic to the level of host specific strain, can confirm the presence of *C. truncatum* on lentil.

7.1.2 General items required

1. Infected plant sample.
2. Compound microscope with 10, 20, 40 and 100x objectives.
3. Dissecting microscope up to 50x magnification.
4. Scalpel blades.
5. Dissecting needle.
6. 70% Ethanol.
7. Sterile distilled water.
8. 2% bleach solution.
9. Sterile petri dishes – 90 x 15 mm
10. Oat meal agar.

7.1.3 Agar plate recipes

½ Strength Oat Meal agar (commerical mix)

Items required

Oat meal agar	36 g
Distilled water	1 L

The above recipe should produce approximately 50 plates.

If a commercial mix of oatmeal agar is not available then the following recipe can be used.

½ strength Oatmeal agar (using rolled oats)

Items required

Oatmeal (rolled oats)	30 g
Agar	20 g
Distilled water	1 L

In a commercial blender, blend 30g of rolled oats in 600ml of distilled water, and heat to 45 – 55°C. Then add 20g agar dissolved in 400ml of distilled water. Autoclave for 90min.

7.1.4 Method - Preliminary examination

- The infected plant material is examined for the presence of microsclerotia or other fruiting bodies under the dissecting microscope. If fruiting bodies are present their identity should be determined under the compound microscope (ie, microsclerotia or pycnidia). If fruiting bodies are found to be pycnidia, the causal pathogen is most likely *Ascochyta lentis* (refer figure 20).
- To be certain of the identity of the pathogen infected plant tissue should be plated onto agar media. Half strength oatmeal agar will induce sporulation in *Colletotrichum truncatum.*

7.1.5 Isolation from infected plant material

1. Infected plant pieces should be cut using a scalpel from the edge of the lesion. Old infected tissues from the centre of the lesions often contain secondary organisms, which invade and colonise the dying tissue. These often include bacteria. Any fruiting bodies that are present should also be plated onto agar media.
2. Tissue pieces to be plated out should be briefly sterilised in 2% bleach solution to remove any surface contaminants. Tissue should be fully submerged in the solution for at least 60 seconds, and up to 2 minutes, time will vary according to the tissue type (ie stem or leaf material) and the size of the piece. Following submergence in bleach, the tissue pieces should be rinsed in sterile distilled water and allowed to dry on clean filter paper before placing on agar plates.
3. Plates should be sealed with parafilm incubated at 18 – 22^0C for 7 – 10 days. This allows adequate time for fungi within the tissue pieces to grow onto the agar, and hence allow for identification.
4. Plates should be clearly marked as "QUARANTINE PATHOGEN", and have an identification number marked on the plate which can be traced back to an original specimen and worksheet.
5. It may become necessary to sub-culture from the initial plates within several days of plating out. Often secondary fungi are fast growing and may contaminate other cultures growing on the plate making sub-culturing necessary to maintain a pure culture. If this needs to be done ensure that the plates are clearly labelled and noted on the specimen worksheet.
6. Once conidia develop, the morphological features of the specimen can be compared to a known description of *Colletotrichum truncatum.* Sutton (1980) provides an excellent morphological description (see section 3.9.1), which can be used to identify suspect cultures suspected to be *C. truncatum*. Also refer to figure 28 for conidia and appressoria illustrations.

8.0 Images

Figure 8. Tan coloured *C. truncatum* lesions of variable size develop on the lower lentil leaflets and the most severely affected leaflets die and drop to the ground. (Photograph courtesy of L. Buchwaldt, Agriculture and Agri-Food Canada.).

Figure 9. Early stages of leaf lesion development by *Colletotrichum truncatum* on lentil.

Figure 10. Early stages of lentil anthracnose leaf lesion development by *Colletotrichum truncatum* on lentil. (Photograph courtesy of R. Morrall, University of Saskatchewan).

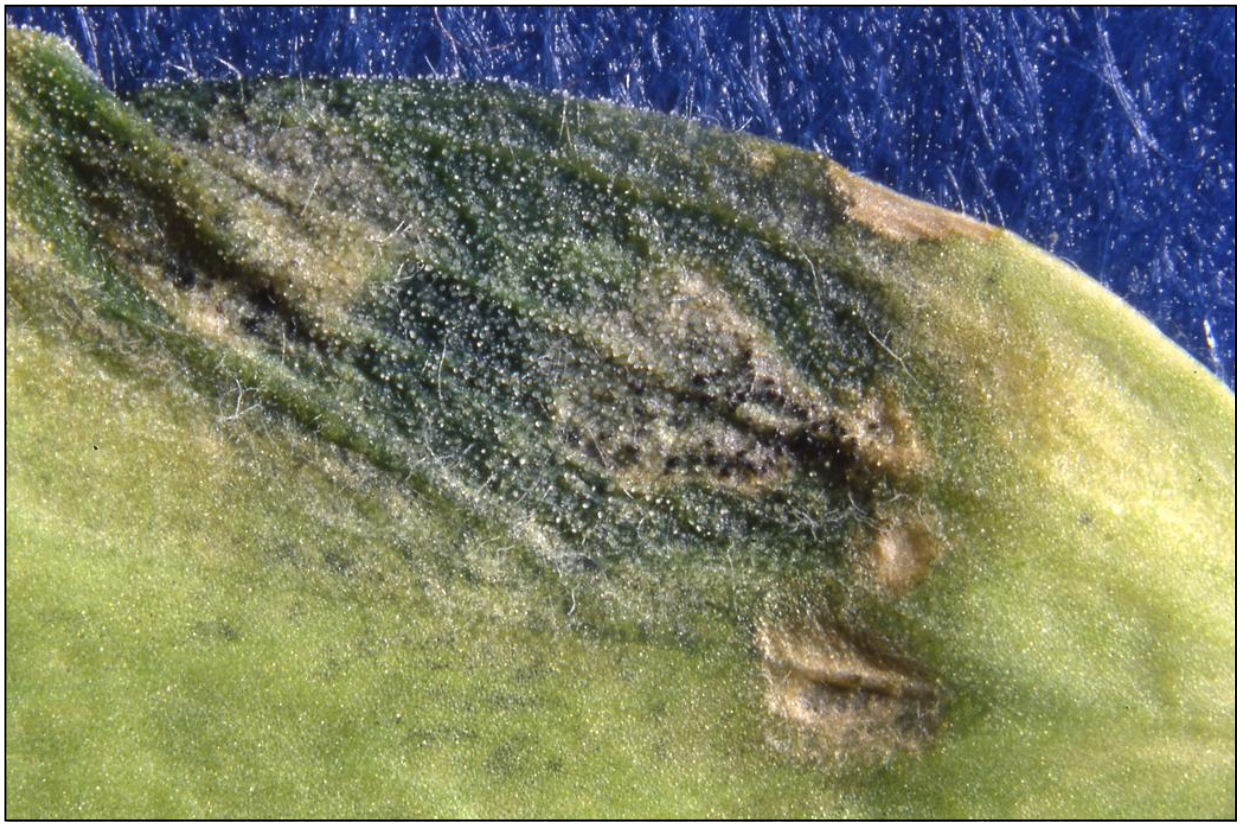

Figure 11. A maturing *C. truncatum* leaf lesion with spore masses and microsclerotia present on a lentil leaf.

(Photograph courtesy of R. Morrall, University of Saskatchewan).

Figure 12. A mature *C. truncatum* leaf lesion with fully formed microsclerotia present on a lentil leaf. (Photograph courtesy of R. Morrall, University of Saskatchewan).

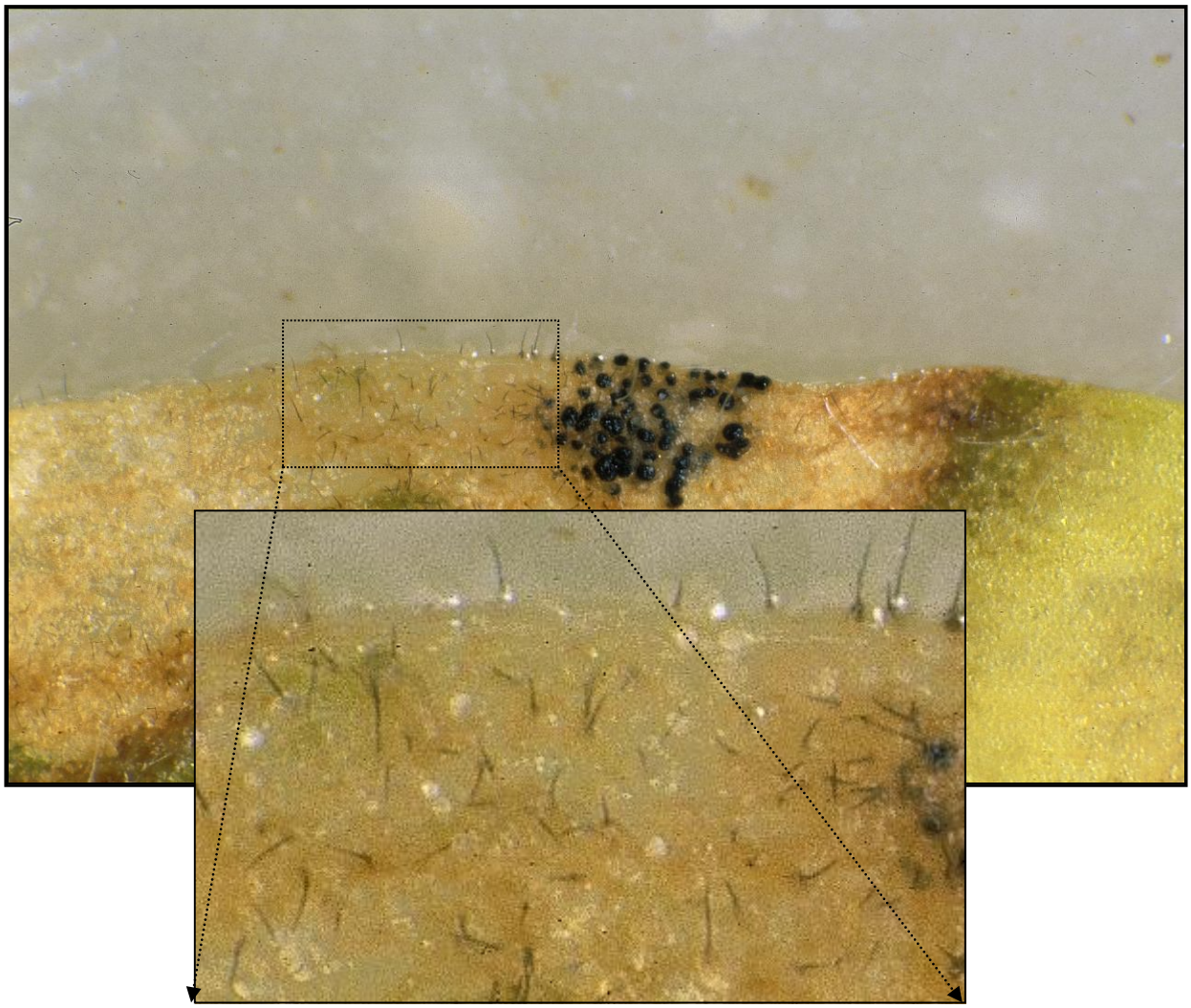

Figure 13. Close up image of a *C. truncatum* stem lesion on lentil showing setae and microsclerotia development. (Photograph courtesy of L. Buchwaldt, Agriculture and Agri-Food Canada.).

Figure 14. A heavily infected lentil plant with *C. truncatum* showing numerous stem lesions. Stem lesions may be small, brownish with a black border, or larger, stretching along the stem. (Photograph courtesy of L. Buchwaldt, Agriculture and Agri-Food Canada.).

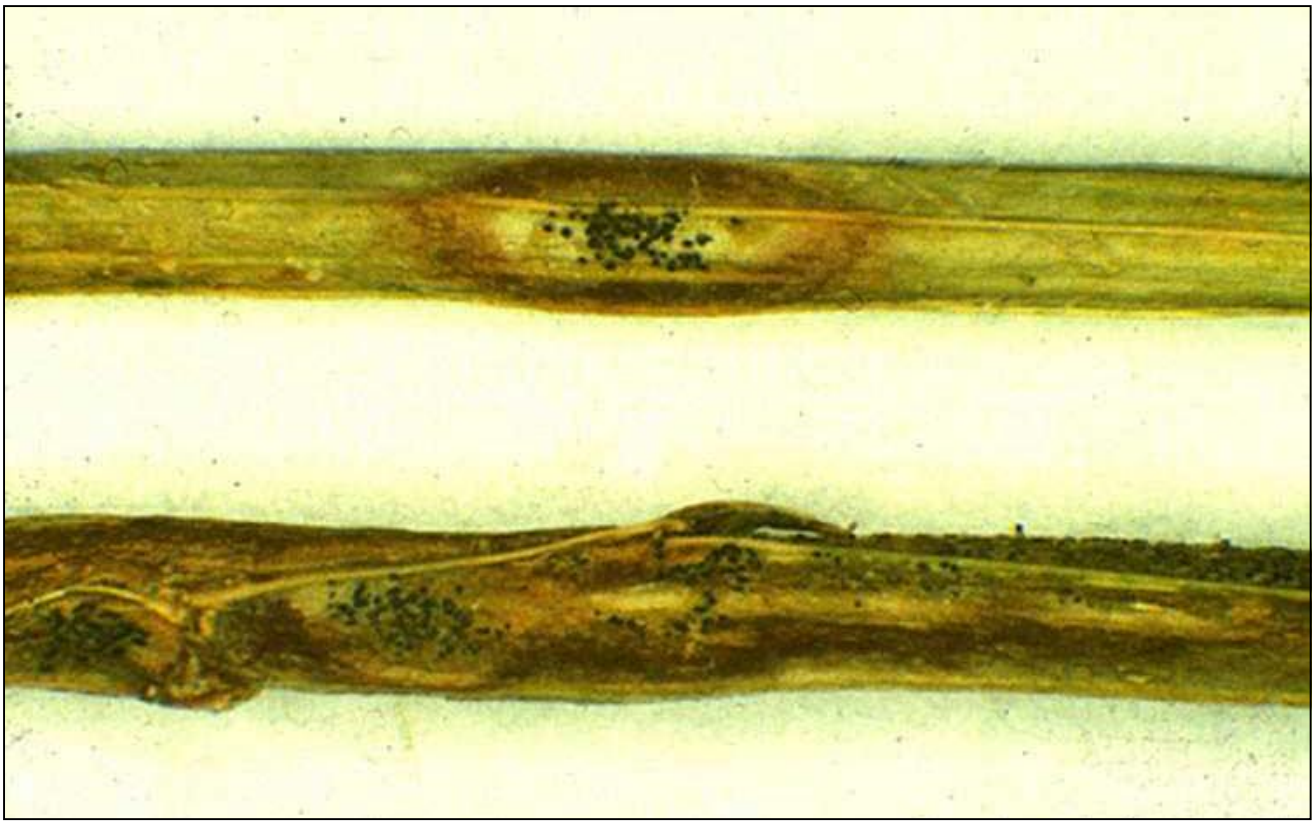

Figure 15. An image of the small, pinhead sized fungal structures (microsclerotia) that form within *C. truncatum* stem lesions on lentil. (Photograph courtesy of L. Buchwaldt, Agriculture and Agri-Food Canada.).

Figure 16. Early lentil anthracnose stem lesion development by *C. truncatum* on lentil in the field. Note the development of microsclerotia within the lesions.

Figure 17. Early *C. truncatum* stem lesion development on lentil prior to the appearance of microsclerotia. (Photograph courtesy of G. Chongo, Agriculture and Agri-Food Canada.).

Figure 18. Stem lesion development by *C. truncatum* infection on lentil.

Figure 19. Detail of *C. truncatum* microsclerotia embedded in a stem lesion on lentil. (Photograph courtesy of G. Chongo, Agriculture and Agri-Food Canada.).

Figure 20. Leaf lesion caused by *Ascochyta lentis* on lentil. Pycnidial fruiting bodies are clearly seen within the lesion. These are not formed by *C. truncatum* on both stem and leaf lesions (Photograph T. Bretag, Dept. of Primary Industries – Victoria).

Figure 21. Under favourable conditions spore masses of *C. truncatum* will form within stem lesions on lentil. These are generally white to pale orange in colour. Rainsplash will disperse these spores and form further infections.

Figure 22. Sporulation at the base of infected lentil plants. These disease symptoms are not considered to be diagnostic as infection by *C. truncatum* or *Fusarium* spp. can produce similar symptoms on lentil.

Figure 23. Large clouds of dust are generated during harvesting of lentil. This photo taken in Canada shows how a crop debris infested by anthracnose can be carried into the air where the wind can blow it into nearby fields or even further. (Photograph courtesy of L. Buchwaldt, Agriculture and Agri-Food Canada.).

Figure 24. Leaf lesions on faba bean caused by *Colletotrichum truncatum* infection. (Photograph courtesy of R. Morrall, University of Saskatchewan.).

Figure 25. A lentil crop heavily infected by *Colletotrichum truncatum* in Manitoba, Canada in 1989. (Photograph

Figure 26. Scattered dying plants within a lentil crop caused by *Colletotrichum truncatum* infection near Regina, Canada 2005.

Figure 27. Small patches or "hotspots" of *C. truncatum* infection forming within a lentil crop near Regina, Canada 2005.

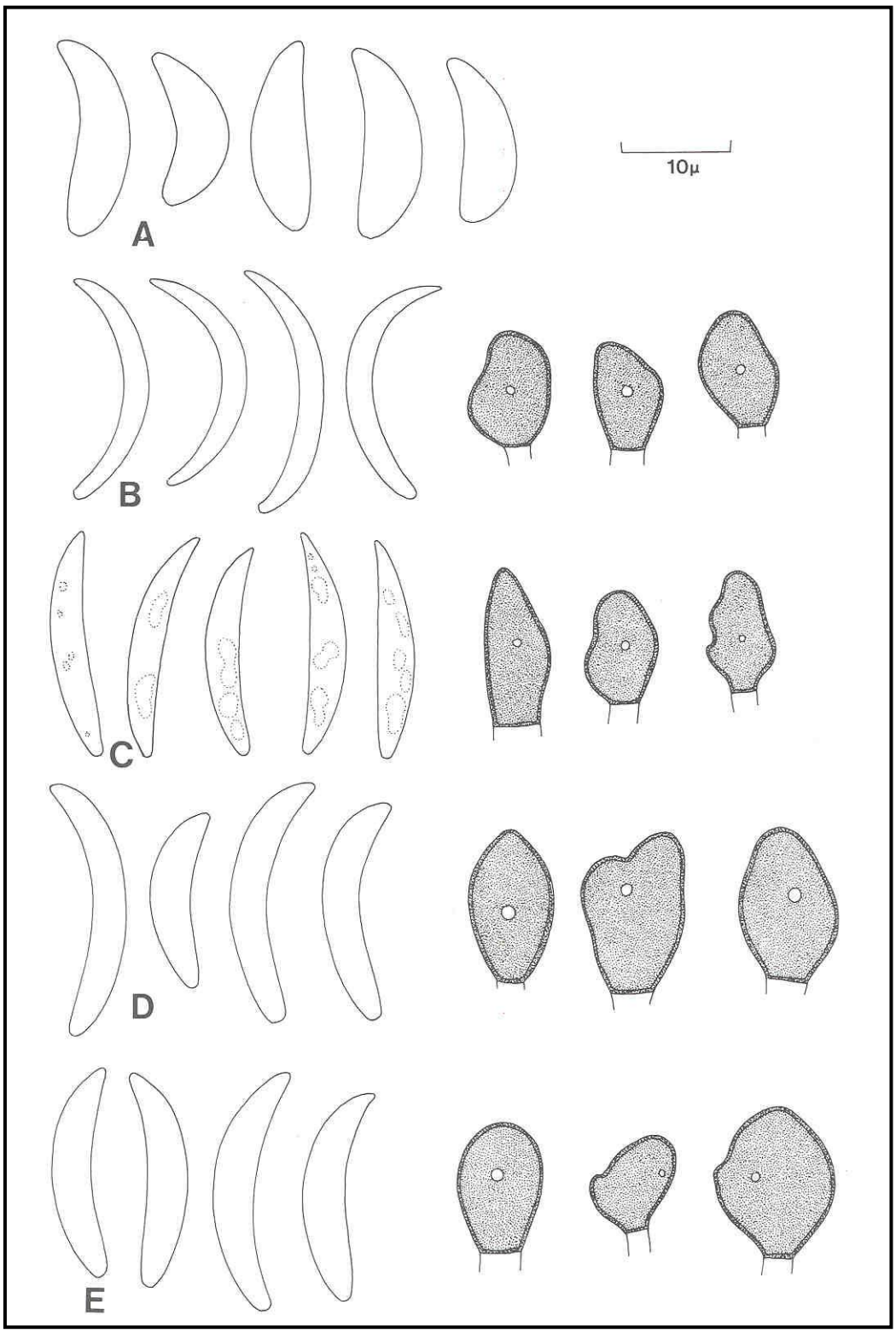

Figure 28. Conidia and appressoria of a number of *Colletotrichum* species that may be mistaken for *C. truncatum*. These include **A**, *Colletotrichum phyllachoroides*, conidia; **B**, *C. dematium*, conidia and appressoria; **C**, *C. truncatum*, conidia and appressoria; **D**, *C. capsici*, conidia and appressoria; **E**, *C. circinans*, conidia and appressoria (taken from Sutton 1980).

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Department of Agriculture, Fisheries and Forestry
http://www.daff.gov.au/content/output.cfm?ObjectID=EDD1F5A3-1E92-4A02-B63340794ACEB370

10.0 Appendices

APPENDIX 1: **Preliminary Information Data Sheet** (Plantplan, 2004).

Date: / / **Sample No.**

SUBJECT

Site details:

Ownership:

Location:

Map (lat. & long.):

GPS identifier:

Host plant location (clearly mark plant if necessary):

HOST DETAILS

Species and variety:

Age:

Developmental stage:

DAMAGE

Description of symptoms:

Part of host affected:

Percent incidence:

Percent severity:

DETAILS OF WHEN AND WHERE THE PEST WAS FIRST NOTICED:

RECORDS OF PRODUCT MOVEMENT ON AND OFF DETECTION SITE:

SYMPTOMS / PHOTOGRAPHS:

FURTHER DETAILS OR COMMENTS:

APPENDIX 2: Questionnaire for trace back of lentil seed

1. Did you purchase certified lentil seed within the last five years? YES/NO

   If the answer was YES go to question 2

   If the answer was NO go to question 6

2. Where was this seed produced? _____ (Name of district)

3. Where did you purchase this seed?

   _____ (Name/address of supplier)

4. Did you record any identifying code numbers or keep any certification tags from the bags of seed? (Seed section can trace a seed source given these details).

   _____

   _____

   _____

   _____

   _____

   _____

5. When did you purchase this seed? Give details

6. Did you purchase uncertified lupin seed within the last five years? YES/NO

   If the answer was YES go to question 7

   If the answer was NO go to question 10

7. From whom did you purchase this seed? (Please give contact details)

   · Local farmer

   · Local merchant

   · Interstate source

8. When did you purchase this seed? (Year)
9. Do you know where this seed was grown? Please give details

10. Have any crops been established from this seed previously? YES/NO

If the answer was YES, go to Question 11

If the answer was NO, there are no further questions

11. Did any of the crops referred to at question 10 grow poorly? Were they harvested? Give any details

12. Further Comments:

APPENDIX 3: Recording sheet for sample receival.

Sample Number. (off Preliminary Data Sheet)**:**

Date of receival:

Receiving laboratory:	**State:**
Receiving Officer:	**Position:**

Host plant (common name):

Botanical name:

Sample plated onto agar? YES/NO	**If Yes, Plate Number:**
Sub sample taken for storage? YES/NO	**If Yes, sub sample number:**

Results from Preliminary examination:

Results from PCR confirmation tests:

Results from morphological examination:

APPENDIX 4: Personnel Hygiene

On **entering** the paddock, personnel **must** :

- Wear protective overalls and rubber boots.
- Prepare footbath of bleach, and spray bottles of methylated spirits brew (95% metho, 5% water) for use following completion of the inspection.
- Conduct inspections by foot (refer to **Appendix 5 Machinery Hygiene** for vehicle access).

On **leaving** the paddock, personnel **must** :

- Wash boots in footbath of disinfectant (solution of household bleach 10%) and remove adhering material, ie soil, with a suitable brush (ie domestic scrubbing brush).
- Spray boots with methylated sprits brew until soaked .
- Remove overalls and place into a bag and seal.
- Exterior of sample bags to be sprayed/swabbed with methylated spirits brew.
- Spray hands with methylated spirits brew irrespective of whether disposable gloves have been worn.

You **must** decontaminate before leaving the paddock **always**.

Overalls must be washed and allowed to completely dry before being used again. If disposable overalls are used, they can be either washed, or if disposed, sent to land fill or burnt.

APPENDIX 5: Machinery Hygiene

- No machinery, including vehicles, are to enter paddock without prior approval from the applicant. Approval to use vehicles in paddock must be included with the application for access.
- Decontamination procedures **must be followed immediately** before leaving the site at the area identified for decontamination.
- Decontaminate the machinery by removing all visible trash and wash down with a high pressure spray using detergent, paying particular attention to the underside, axles, wheels and tyres. This also includes all hand held tools such as hoes and shovels.
- Personal decontamination procedures must follow the decontamination of machinery.
- It is recommended that any machinery or vehicle that has entered the paddock is not to be taken into another green lentil crop this season.

Harvest Machinery

- In addition to the above requirements, machinery will be cleaned of all seed and trash remaining. This material will be destroyed in a manner approved by the relevant State Authority (ie, landfill within quarantine boundary or similar).

APPENDIX 6: Solutions/Reagents and Suppliers

6.1. DNA Extraction

Extraction buffer (pH 5.5) (refer Ford *et al.*, 2004).

	1L	Final concentration
Cetyltrimethyl ammonium bromide (CTAB)	20.0 g	2% w/v
NaCl	81.82 g	1.4 M
0.5M EDTA (pH 8.0)	40 mL	20 mM
1.0M Tris HCl (pH8.0)	100 mL	100 mM

- Dissolve solids and mix solutions in 1L sterile RO water.
- Autoclave before use.
- Store at room temperature.
- Note: EDTA is a hazardous substance. Read MSDS before use

1.0M Tris HCl (pH8.0)

- 12.1g Tris base dissolved in 100 ml sterile RO water
- pH to 8.0 with concentrated HCl
- autoclave before use
- store at room temperature

0.5M EDTA (pH8.0) ethylenediamine trisodium acetate

- 18.6g EDTA dissolved in 100ml sterile RO water
- pH to 8.0 with NaOH while stirring. Solid will not dissolve until close to pH8.0
- autoclave before use
- store at room temperature

TE Buffer

	1 L	Final concentration
1M Tris HCl (pH 8.0)	10 mL	10 mM
0.5M EDTA (pH 8.0)	2 mL	1 mM

- Mix reagents in 1L sterile RO water
- Autoclave before use
- Store at room temperature

Phenol:chloroform:isoamyl alcohol. (25:24:1).

- Buy solution already mixed in these ratios
- Store at 4^{o}C

NB. Phenol is a carcinogen and appropriate gloves, safety glasses and laboratory coats must be worn at all times. Phenol must always be used in a fume hood.

10mg/ml RNAse A

	10 mL	Final concentration
RnaseA	100 mg	10 mg/mL
1M Tris-HCl pH7.5	100 μL	10 mM
5M NaCl	3 μL	15 mM

- Dissolve components in 10 mL sterile RO water
- Boil for 15 min
- Cool slowly to room temperature
- Aliquot into 1mL volumes
- Store at –20^{o}C

6.2 Traditional PCR Reagents

PCR reagents

- Taq DNA Polymerase recombinant reagent kit available from Invitrogen (Catalogue number – 10342-020).

It contains:

- 10x PCR buffer minus Mg
- 10mM dNTP mixture
- Taq polymerase (5units/μl)
- 50mM MgCl

Mix of 20mM each dNTPs

5x TBE Buffer

	1 L	Final concentration
Tris base	54.0 g	0.4 M
Boric acid	27.5 g	0.05 M
0.5M EDTA pH 8.0	20.0 mL	0.001 M

- Dissolve components in 1 L sterile RO water
- Store at room temperature.

1.4% Agarose with ethidium bromide

	100 mL	Final concentration
DNA grade agarose	1.4 g	1.4%
1 x TBE	100 mL	1x
10mg/ml Ethidium bromide	3 μL	0.3 mg/mL

- Dissolve agarose in TBE by heating in microwave for 1 min 20 sec
- Mix by shaking
- Cool to hand temperature and add Ethidium Bromide, mix
- Pour into get tray with comb to set

NB. Phenol is a carcinogen and appropriate gloves, safety glasses and laboratory coats must be worn at all times. Phenol must always be used in a fume hood.

6x Loading dye

	50 mL
1 x TE	5 mL
Glycerol	25 mL
bromophenol blue	0.125
xylene cyanoll FF*	0.125

- Make up in 50 ml RO water
- Store at room temperature

***NB.** xylene cyanoll FF is an irritant (in powder form)

6.3 Real – time PCR Reagents

Master Mix

Brilliant® SYBR® green QPCR master mix (Stratagene #600548)

6.4 Suppliers and catalogue numbers

Chemical	Supplier	Catalogue #
Agarose	Progen	2000011
Boric acid	Sigma	11611
Bromophenol blue	Sigma	B-5525
CTAB	Amresco	0883
EDTA	Sigma	43178-8
Ethanol	Merck	10476.9020
Ethidium bromide	Merck	443922U
Glycerol	Sigma	G6279
HCl	Merck	10307 6P
NaCl	Merck	1024.3
Phenol/chloroform/isoamyl alcohol	Merck	43673 4C
Real-time PCR "Master Mix" Brilliant® SYBR® green QPCR master mix	Stratagene	600548
RnaseA	Sigma	R4875
Taq DNA Polymerase reagent kit	Invitrogen	10342-020
Tris base	Calbiochem	648317
Xylene cyanol FF	Aldrich	33594-0