# Investigation of the cause of 'Black spot' disorder of avocado fruit in Peru

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In Peruvian 'Hass' avocado fruit, a cosmetic disorder termed 'black spot' may appear after 2–3 weeks in coolstorage, causing brown to black blotches usually 2–3 cm in diameter on green fruit. This disorder may be caused by fungal infection, physical damage, lenticel breakdown, water loss, chilling injury or a combination of these factors. A series of experiments was conducted in Peru over several seasons and in different climatic zones to investigate the etiology of this disorder. Results of isolations from 'black spot' symptoms and adjacent green tissue on the same fruit suggest that there are probably a number of symptom types that are called 'black spot' that are probably related to physical damage, fungal invasion and chilling injury. The evidence for fungal invasion needs to be further examined by Koch's postulates.

## INTRODUCTION

'Black spot' is a quality issue identified as a potential problem for 'Hass' avocado fruit exported from Peru. The 'black spot' symptoms visible after storage are variable; some are similar to severe lenticel damage and others are possibly caused by fungi. The cause of the 'black spot' symptoms is not known and could be pathological, physical or physiological in origin. Studies from elsewhere have shown that lenticel damage is a physiological disorder (Everett *et al.,* 2008). In New Zealand there is another disorder, called 'measles', that shows symptoms similar to 'black spot' in Peru. Originally 'measles' was hypothesised to develop from lenticel damage. Instead 'measles' was shown to be associated with pathogenic fungi and symptoms were worsened by handling damage. 'Measles' were thus a separate disorder from lenticel damage (Everett *et al.* 2008). A similar investigation of 'black spot' was conducted in Peru.

## MATERIALS AND METHODS

A total of 203 fruit showing symptoms of 'black spot' were sourced from library trays of fruit being held in the coolstores of nine companies (Table 1). Fruit are identified by the orchard they came from, with orchards designated C1 to C11. Symptoms on all fruit were photographically recorded. Isolations were made from symptomatic and non-symptomatic skin on every fruit. Fruit were wiped with 70% ethanol before a small piece of skin was aseptically excised with a sterile scalpel blade and placed on Difco® potato dextrose agar (PDA). A symptomless skin sample from the same fruit was sampled in the same way. Six tissue pieces in total were excised from every fruit.

Fungal cultures were identified by morphology, and a sample of representative fungi was sub-cultured and subjected to DNA analysis. Cultures for DNA extraction were grown on PDA for 2–3 weeks at ambient temperature (approximately 20°C). DNA was extracted from approximately 1 cm2 mycelium by using the DNeasy® Tissue kit (Qiagen).

The ITS regions ITS1 and ITS2 and the 5.8S gene were amplified using the primer set ITS1/4 (White *et al.,* 1990) as described in Everett *et al.* (2011). The PCR products were sequenced in both directions by Macrogene Inc. All sequences were edited manually using Vector NTI 8 (InforMax Inc., Bethesda, MD, USA) and ambiguous regions on both sides of the sequences were excluded from the analysis.

A neighbour joining (NJ) analysis (Saitou & Nei, 1987) was performed with Molecular Evolutionary Genetics Analysis (version 6) software (Tamura & Nei, 1993; Tamura *et al.,* 2013), using the heuristic search option, and 1,000 random addition sequence replicates were used. The bootstrap values were evaluated by using 1,000 replicates to test the branch strength (Felsenstein, 1985). A *Puccinia graminis* sequence was used as the outgroup (GenBank accession number JX047482). The Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/blast) was used to find sequences that were homologous to those derived from the isolates from Peru and included in the phylogenetic analysis.

## RESULTS

There were at least three different symptom types of 'black spot' (Figure 1) submitted for isolations.

1. Damaged nodules (brown sunken lesions on protuberances)

Blotches with sharp borders; 'discrete skin discolouration' – Page 32–33 of White *et al.* (2009), possible chilling injury as in Yearsley and Lallu ( 2001)

Blotches with fuzzy borders; 'measles' of Everett *et al.* (2008), external rots – Page 26–27 of White *et al.* (2009).

Symptoms of sunken darkened nodules (Figure 1a) attributed to physical damage were found on fruit from all orchards. Two orchards (C6 and C11) had only these symptoms on their fruit. Some symptoms on fruit from C1 and C4 were different from those on fruit from the other six orchards. These symptoms were of diffuse black/brown blotches (Figure 1c). There were similar symptoms on fruit from the other orchards, but the margins of the black/brown blotches were discrete (Figure 1b), except for in seven out of 29 fruit from C3.

*Cladosporium* sp. was the most common fungus isolated from both 'black spot' symptoms and symptomless skin (Table 1). There were more isolations of *Cladosporium* sp. from 'black spot' symptoms than from symptomless tissue on the two orchards (C1 and C4) that showed symptoms of diffuse black/brown blotches (Figure 1c).

There was no difference in the numbers of isolations from 'black spot' symptoms and from symptomless green skin for the other seven orchards (C2, C3, C5, C6, C8, C9, C11).

Results of phylogenetic analysis confirmed the identification by culture morphology of the fungi isolated from symptomless and 'black spot' skin tissue as *Colletotrichum gloeosporioides*, *Nigrospora* sp., *Lasiodiplodia theobromae*, *Cladosporium* sp. and *Alternaria* sp. (Figure 2). Because the fungi other than *Cladosporium* were isolated in equal numbers from both symptomless green and 'black spot' affected avocado skin (Table 1), these were unlikely to be pathogens, but instead saprotrophs invading the already damaged tissue. It is possible that after coolstorage and ripening the two known pathogens, *C. gloeosporioides* and *Lasiodiplodia theobromae*, may infect the fruit to cause postharvest rots. These fungi were isolated from postharvest rots from these same avocados following ripening (Table 2).

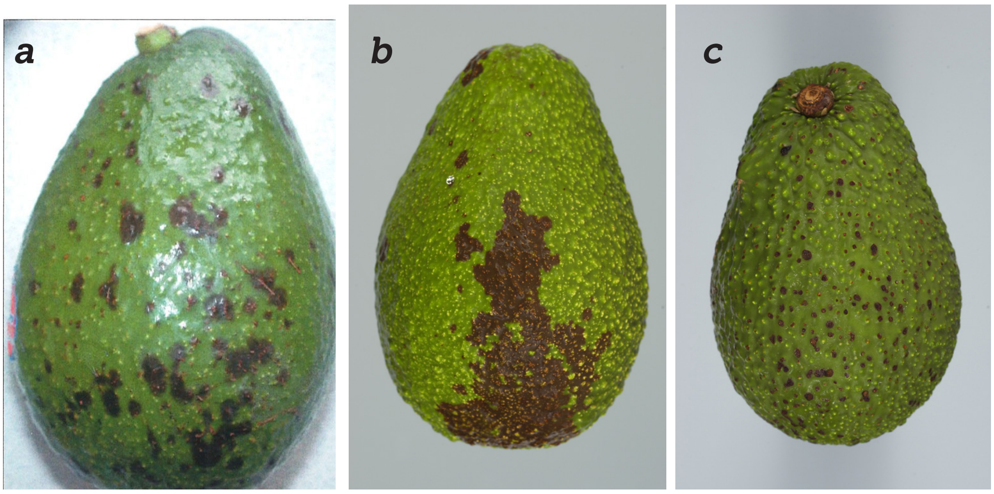


**Figure 1. Symptoms of a) damaged avocado fruit nodules (brown sunken lesions on protuberances), b) chilling injury (blotches with sharp borders) and c) blotches (fuzzy borders).**

**Table 1. Isolations from 'black spot' symptoms and from symptomless green skin on the same Peruvian 'Hass' avocado fruit. Fungal cultures were identified by morphology, and representative isolates were DNA sequenced for confirmation. Three samples were taken from 'black spot' and symptomless skin for each fruit. P values are the result of a chi-squared analysis.**

| | | Isolations from 'black spot' | | | | | | | Isolations from symptomless green skin | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Orchards | No. of fruit | Clad. | Alt. | Cg | LD | Bot | Pen | Total | Clad. | Alt. | Cg | LD | Bot | Pen | Fus. | Nig. | Pest. | Total | P |
| C1 | 33 | 18 | 1 | | 1 | | | 20 | 8 | 1 | | | | | | | | 9 | 0.0499 |
| C2 | 1 | 1 | | | | | | 1 | 2 | 1 | 1 | | | | | | | 4 | n.s. |
| C3 | 29 | 18 | 1 | 4 | | 1 | | 24 | 16 | 2 | 4 | | | | 1 | | | 23 | n.s. |
| C4 | 22 | 38 | | | | | | 38 | 18 | | | | | | | | | 18 | 0.0075 |
| C5 | 5 | 3 | | 2 | | | | 5 | 3 | | | | | | | | | 3 | n.s. |
| C6 | 11 | 23 | | | | | | 23 | 21 | | 1 | | | 1 | | | | 23 | n.s. |
| C8 | 73 | 89 | 3 | 2 | 5 | 2 | | 101 | 87 | 5 | 4 | 4 | 5 | 1 | | 1 | 2 | 109 | n.s. |
| C9 | 15 | 33 | | 2 | | | 1 | 36 | 26 | 2 | 1 | | | | | | | 29 | n.s. |
| C11 | 14 | 15 | 1 | | | | | 16 | 13 | 1 | | | | | | | | 14 | n.s. |
| Total | 203 | 238 | 6 | 10 | 6 | 3 | 1 | 264 | 194 | 12 | 11 | 4 | 5 | 2 | 1 | 1 | 2 | 232 | |

Clad. = Cladosporium, Alt. = Alternaria, Cg = Colletotrichum gloeosporioides, LD = Lasiodiplodia, Bot. = Botrytis, Pen = Penicillium, Fus. = Fusarium, Nig. = Nigrospora, Pest.= Pestalotiopsis. n.s. = not significant

**Table 2. Isolations from rots that developed in ripened Peruvian 'Hass' avocado fruit. Fungal cultures were identified by morphology, and representative isolates were DNA sequenced for confirmation.**

| Orchard | Date harvested | No. of fruit sampled | Ripening time (days) | No. of body rots | No. of stem-end rots | % rots | C.g. | L.t. |
|---|---|---|---|---|---|---|---|---|
| C2 | 1/7/2014 | 67 | 9.8 ± 0.27 | 0 | 0 | 0 | 0 | 0 |
| C3 | 27/6/2014 | 70 | 10.8 ± 0.07 | 0 | 62 | 88.6 | 1 | 60[1.] |
| C4 | 25/6/2014 | 69 | 16.7 ± 0.32 | 1 | 27 | 40.6 | 1 | 18[1.] |
| C5 | 25/6/2014 | 65 | 15.1 ± 0.17 | 9 | 24 | 50.8 | 7 | 24 |
| C6 | 2/7/2014 | 39 | 13.3 ± 0.43 | 2 | 3 | 12.8 | ? [1.] | ? [1.] |
| C8 | 26/6/2014 | 64 | 14.0 ± 0.19 | 0 | 25 | 39.1 | 0 | 23[1]. |
| C10 | 31/6/2014 | 62 | 18.1 ± 0.28 | 0 | 7 | 11.3 | 0 | ?[1.] |
| C11 | 1/7/2014 | 34 | 18.3 ± 0.50 | 0 | 14 | 41.2 | 0 | 1[1.] |
| Total | | 470 | | 12 | 162 | | 9 | 126 |
| % | | | | 2.6 | 34.5 | 37.0 | 7.3 | 92.6 |

1. Fungi were isolated from a fruit sub-sample dependent on whether fruit were ripe during the pathologist visit. C.g. = *Colletotrichum gloeosporioides*, L.t. = *Lasiodiplodia theobromae*.

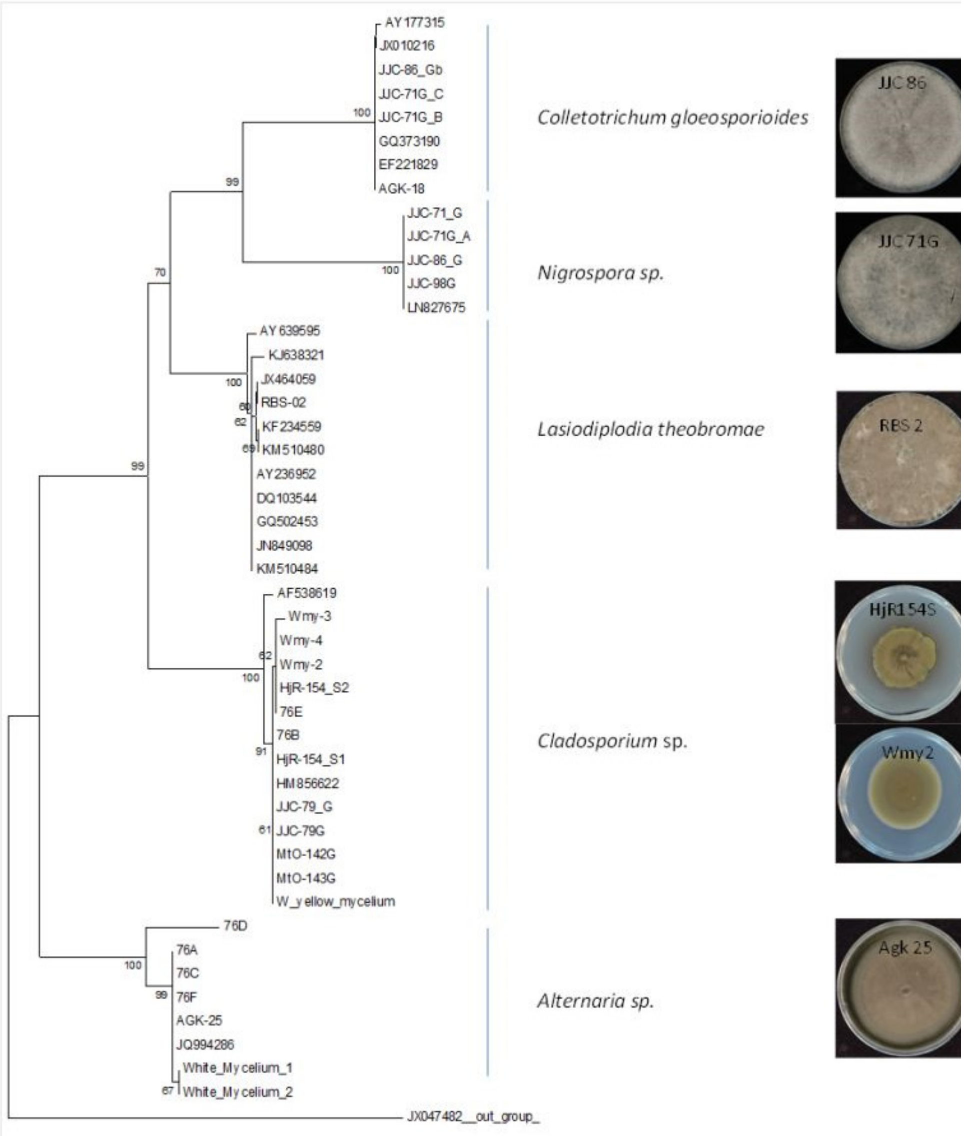


Figure 2. The results of a neighbour-joining phylogenetic analysis using the Tamura-Nei method (Tamura & Nei 1993) for fungi isolated from Peruvian 'Hass' avocado fruit with black spot symptoms. The optimal tree with the sum of branch length = 1.0 is shown. The percentage of replicate trees >55, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site. The analysis involved 47 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 309 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

## DISCUSSION

The three different symptom types need to be clearly distinguished so that appropriate measures can be taken to remediate 'black spot'. Accurate diagnosis of the type of 'black spot' will assist growers, exporters and packhouse managers to make the appropriate modifications to current practice to minimise the number of affected fruit.

Damaged nodules appeared to be a common problem on fruit from all orchards. Modifications to the harvesting procedures may avoid this issue. For instance, ensuring that the roads on which the fruit are transported are smooth without unnecessary bumps, that the means of offloading the fruit onto the packing line are as gentle as possible, and that there are as few drops or impacts as possible in the packing lines.

Chilling injury occurs when the fruit are cooled to a temperature that is too low (generally less than 3°C; White *et al.* 2009). This critical temperature for fruit may change during the season, and may be different for fruit from different climatic zones. Therefore the ideal storage temperatures for Peruvian 'Hass' avocados from different climatic zones may need to be determined, and then be rigorously adhered to.

For two orchards (C1 and C4), there were more isolations of the fungus *Cladosporium* from surface-sterilised 'black spot'-affected tissue than there were from adjacent 'green' tissue on the same fruit. There was no difference of numbers of fungal isolations from 'black spots' and green tissue for fruit from the other sampled orchards. This evidence from isolations suggests that 'black spot' on fruit from these two orchards may be associated with a fungal pathogen (*Cladosporium* cladosporioides).

*Cladosporium* has been reported to cause decay or surface moulds on melons (Suslow *et al.,* 1997), but elsewhere the primary cause was attributed to physical or chilling injury, and colonisation by *Cladosporium* was as a secondary invader (Zitter *et al.,* 1996). Its status on avocados in Peru is not known. It would require testing Koch's Postulates (Agrios, 1997) to determine if *Cladosporium* was a pathogen of avocado.

## ACKNOWLEDGEMENTS

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# ACTAS · PROCEEDINGS

## VIII CONGRESO MUNDIAL DE LA PALTA 2015

del 13 al 18 de Septiembre. Lima, Perú 2015

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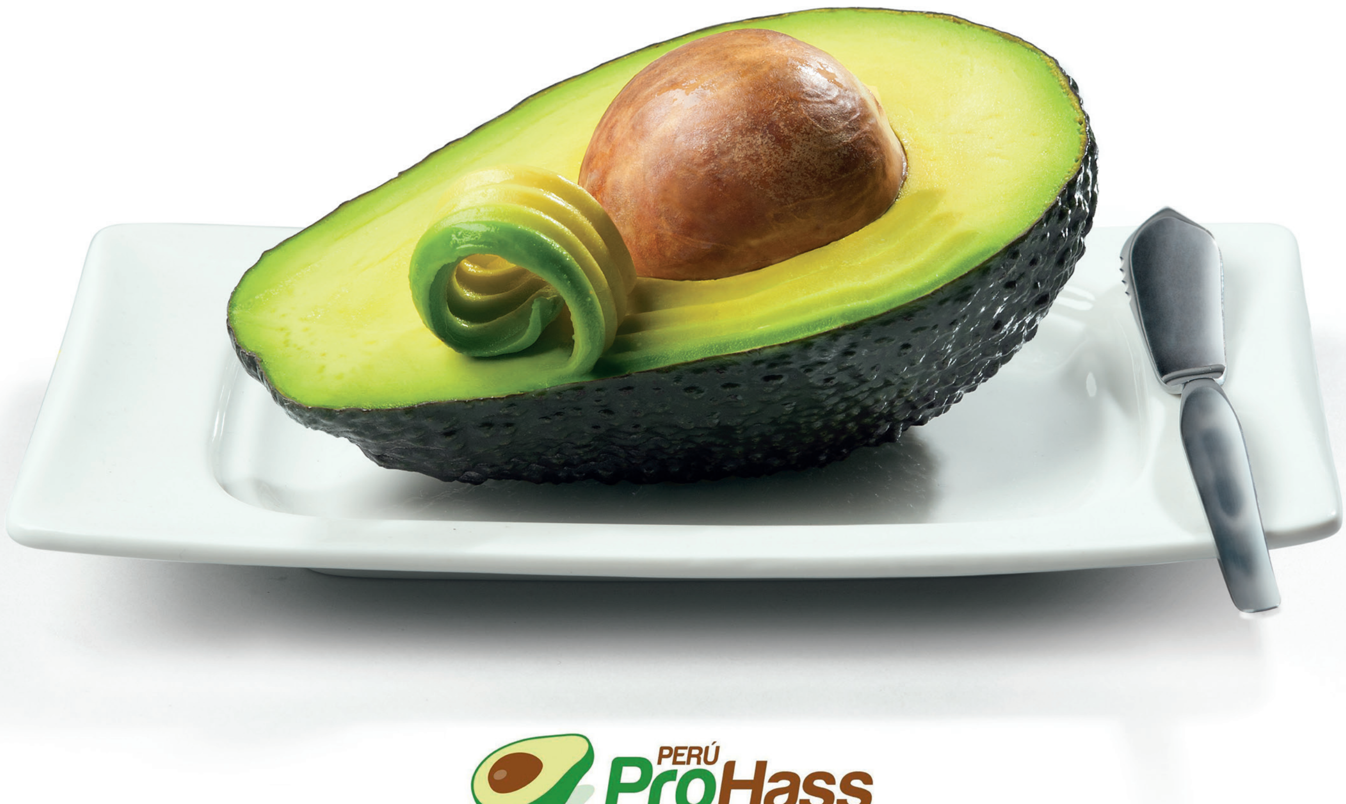

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