



# Non-invasive detection of chestnut (*Castanea sativa* L.) rot caused by *Gnomoniopsis smithogilvyi*

Stage 1 - Proof of concept

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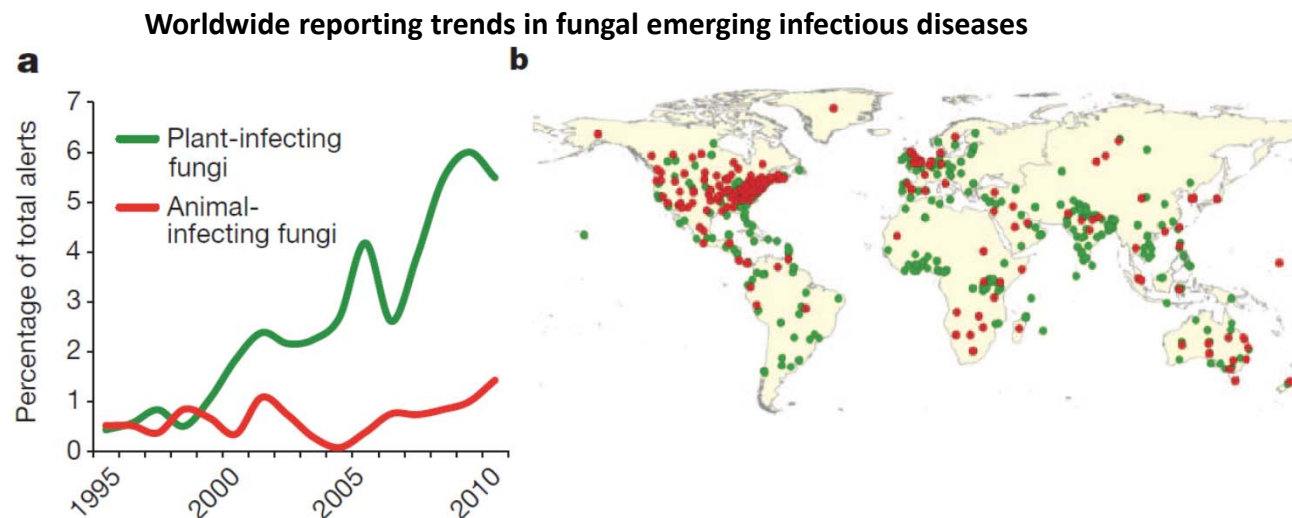
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# Fungal plant diseases

- Fungi constitute the largest number of plant pathogens and are responsible for a range of serious plant diseases.
- Fungi damage plants by killing cells and/or causing plant stress.
- Sources of fungal infections are infected seed, soil, crop debris, nearby crops and weeds.
- Fungi are spread by wind and water splash, and through the movement of contaminated soil, animals, workers, machinery, tools, seedlings and other plant material.
- Fungi enter plants through natural openings such as stomata and through wounds caused by pruning, harvesting, hail, insects, other diseases, and mechanical damage.



# Chestnut

## *Background information*

- The main species grown in Australia are:
  - *Castanea sativa* (European chestnut) and
  - hybrids of *C. crenata* × *C. sativa* (Japanese chestnut × European chestnut)
- Chestnuts are grown in cooler areas of Australia such as –
  - North-East Victoria (VIC) – accounting for 80 % of the Australian crop is grown
    - The Dandenong Ranges
    - Gippsland
    - the Macedon Ranges
  - New South Wales (NSW) -
    - the Blue Mountains
    - Central-West NSW
    - the North Coast of NSW
    - the South Coast of NSW.
- South Australia & Tasmania and Western Australia also grow small quantities



<https://www.chestnutsaustralia.com.au/chestnut-info/>

Shuttleworth *et al.* (2013). Australasian Plant Path. 42:63-72

# Chestnut

## *Background information*

Chestnut rot is a significant problem in the Australian chestnut industry –

- It affects the chestnut kernel, symptoms occur as pale, medium and dark brown lesions occurring on the endosperm and embryo
- 22 orchards in Victoria and New South Wales were surveyed in 2008, and 21 of these were again surveyed in 2009 -
  - All 22 orchards were affected by chestnut rot
    - Incidence at individual orchards up to 72 % was found in 2008, and
    - 35 % in 2009.
  - Incidence varied widely between orchards and within individual orchards between the 2 years
    - All varieties were affected, including Decoppi Marone, Purtons Pride and Red Spanish, displaying examples of both high incidence (>1 %) and industry acceptable incidence (0–1 %) depending on orchard and year.
    - In 2008 and 2009, surveys of Sydney Markets showed incidence of >1 % (2008: varieties Decoppi Marone, Purtons Pride; and in 2009: varieties Purtons Pride, Red Spanish).
  - The early December flowering Red Spanish had significantly higher average incidence than the early to mid December flowering Decoppi Marone and Purtons Pride, suggesting that the timing of flowering may be an important factor affecting incidence.

# Chestnut rot - Background



The disease causing fungi

- *Gnomoniopsis smithogilvyi* sp.



Fungi exist in the orchard

- *saprophyte- decaying burrs*
- *chestnut flowers, leaves and stems*
- *perithecia*
- *ascospores - primary source of inoculum*



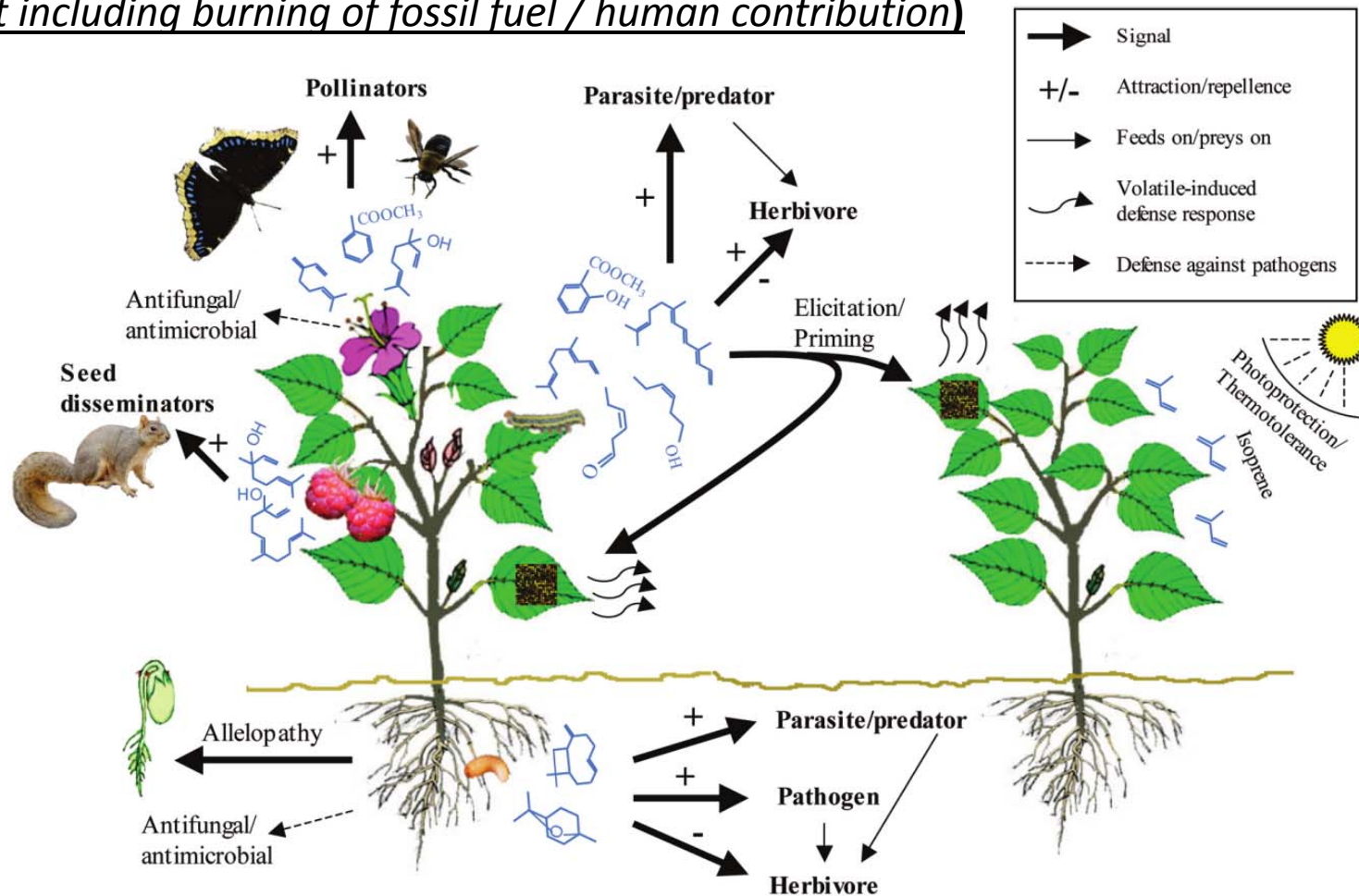
Reducing the incidence of chestnut Rot

- *Orchard hygiene*
- *Biological control*
- *Variety selection*

Source: Pest and disease information Bulletin, Chestnut Australia Inc

# Non-invasive detection technology

Sources of volatile organic compounds in nature  
 (not including burning of fossil fuel / human contribution)



# Non-invasive detection technology

Volatiles are the group of chemical compounds with low boiling points:

- Distinct volatiles emanate from:
  - Undamaged plants
  - Plants damaged by herbivore / microbes (bacteria, yeast or mold) / insects
- This implies stimulation of biosynthetic pathways
- Factors affecting amount or condition
  - Degree of injury
  - Behavior of insects when attacking plant
- Factors affecting blend of a volatile compound
  - Type of insect / microbes
  - Juvenile or adult insect stages

# Non-invasive detection technology

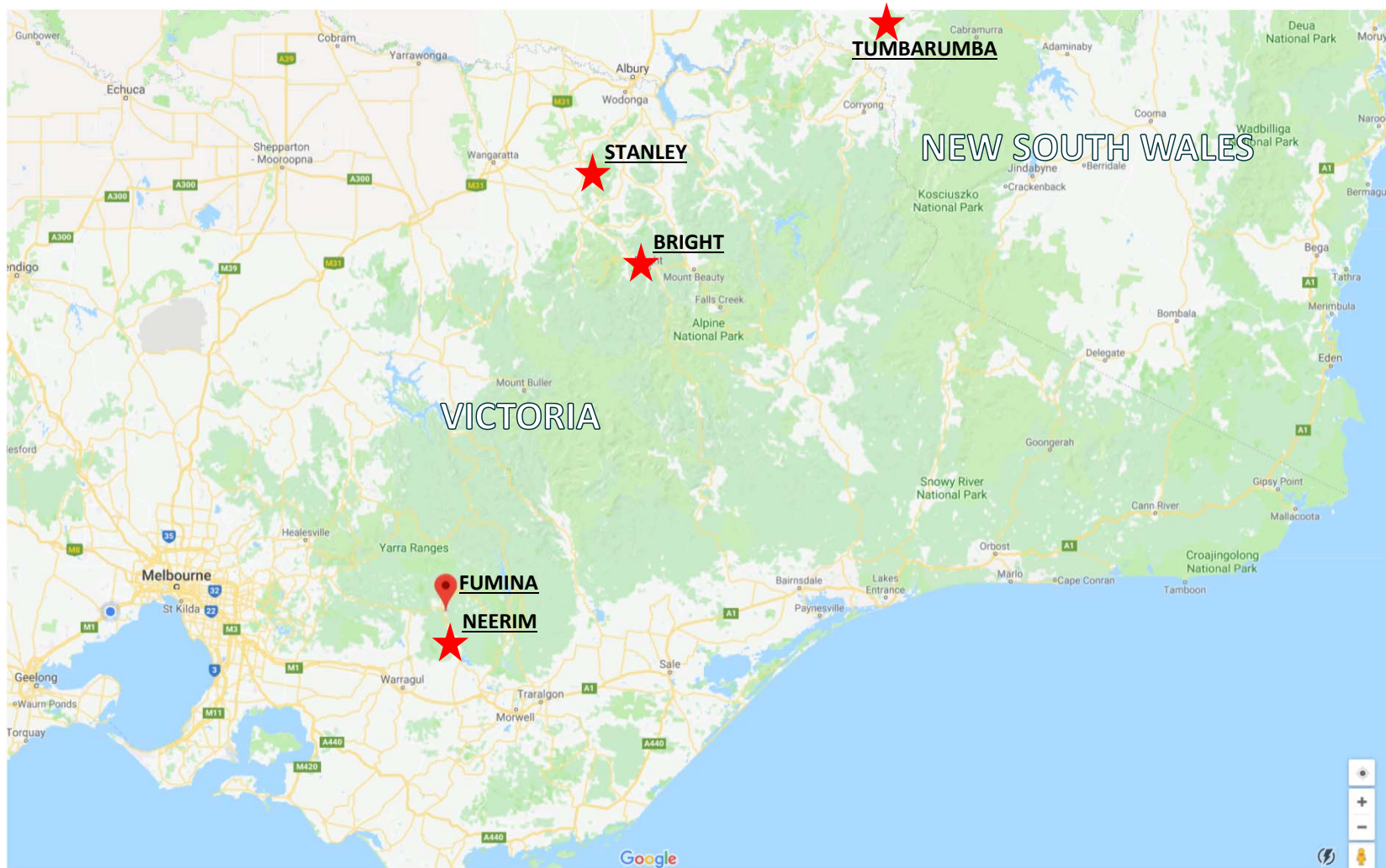
- Volatiles secreted in minutes after tissue injury
- Damage / injury could potentially stimulate extra-floral nectar release in non-injured parts of injured plants
- Stimulates other local plants
- Gaseous nature of the volatiles speeds communication in the damaged plant itself
  - MORE RAPID MOVEMENT THAN AN INTERNAL COMMUNICATION SYSTEM
- Type of compounds
  - Aldehydes
  - Alcohols
  - Esters
  - Terpenoids.....



# Chestnut samples

Chestnut / burr samples	Details from package / observation	Chestnut Received / Region
Chestnut burr	Small brown spots on burr	Collected at an orchard- Bright (VIC) Feb 2017
Purtons		Aug - 2017; Stanley (VIC)
Menzies		Aug - 2017; Stanley (VIC)
Menzies	one infected (brown/black patches) chestnut - infected	Aug - 2017; Stanley (VIC)
Purtons Pride		Sep - 2017; Fumina (VIC)
Easy Peel Me		Sep - 2017; Fumina (VIC)
Me	stored at room temperature	Sep - 2017; Neerim (VIC)
P Gold L3	stored at room temperature	Sep - 2017; Neerim (VIC)
		Sep - 2017; Tumbarumba (NSW)

# Origins of chestnut samples



# Experimental details

- Volatiles emanating from chestnut / fungal cultures were sampled by solid phase micro-extraction fibre (SPME fibre; coated with CAR/PDMS) at 30°C
- Extracted volatiles were desorbed directly into hot PTV GC inlet (260°C, set in splitless mode, 1 min)
- Volatiles were chromatographed on a WAX MS column using a temperature gradient
- Eluted compounds were detected and identified by mass spectrometry



Glass vessel



SPME fibre/holder assembly

# Non-invasive detection of fungal plant disease

## *Chestnut burr with brown spots*

- Chestnut burr samples, with and without brown discolouration, were collected at an orchard located in Bright (VIC) area
  - Brown discoloured patches were excised and plated on microbiological plates
    - Mould growing on the plates were re-plated on microbiological media and investigated for volatile compound(s) production (*see next two slides for further information*)

# Non-invasive detection of chestnut rot

## *Chestnut burr with brown spots*



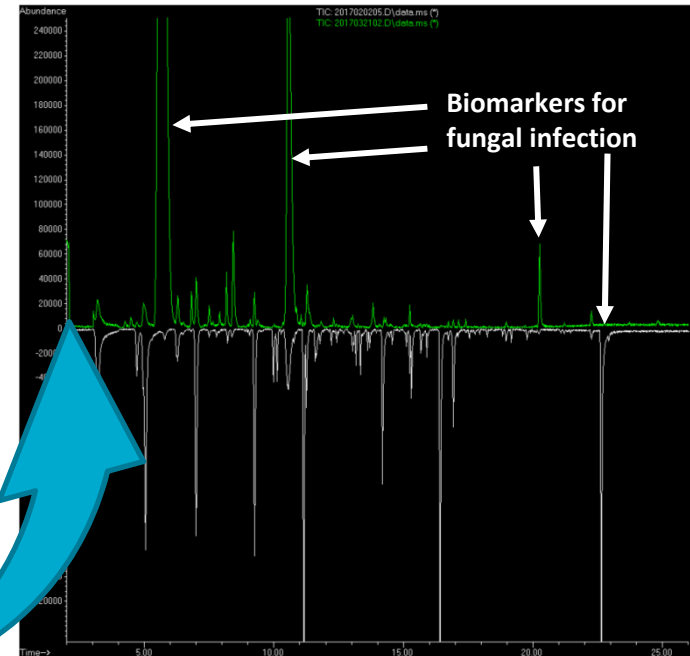
**Chestnut orchard  
Bright (VIC)**



**Infected Chestnut burr**

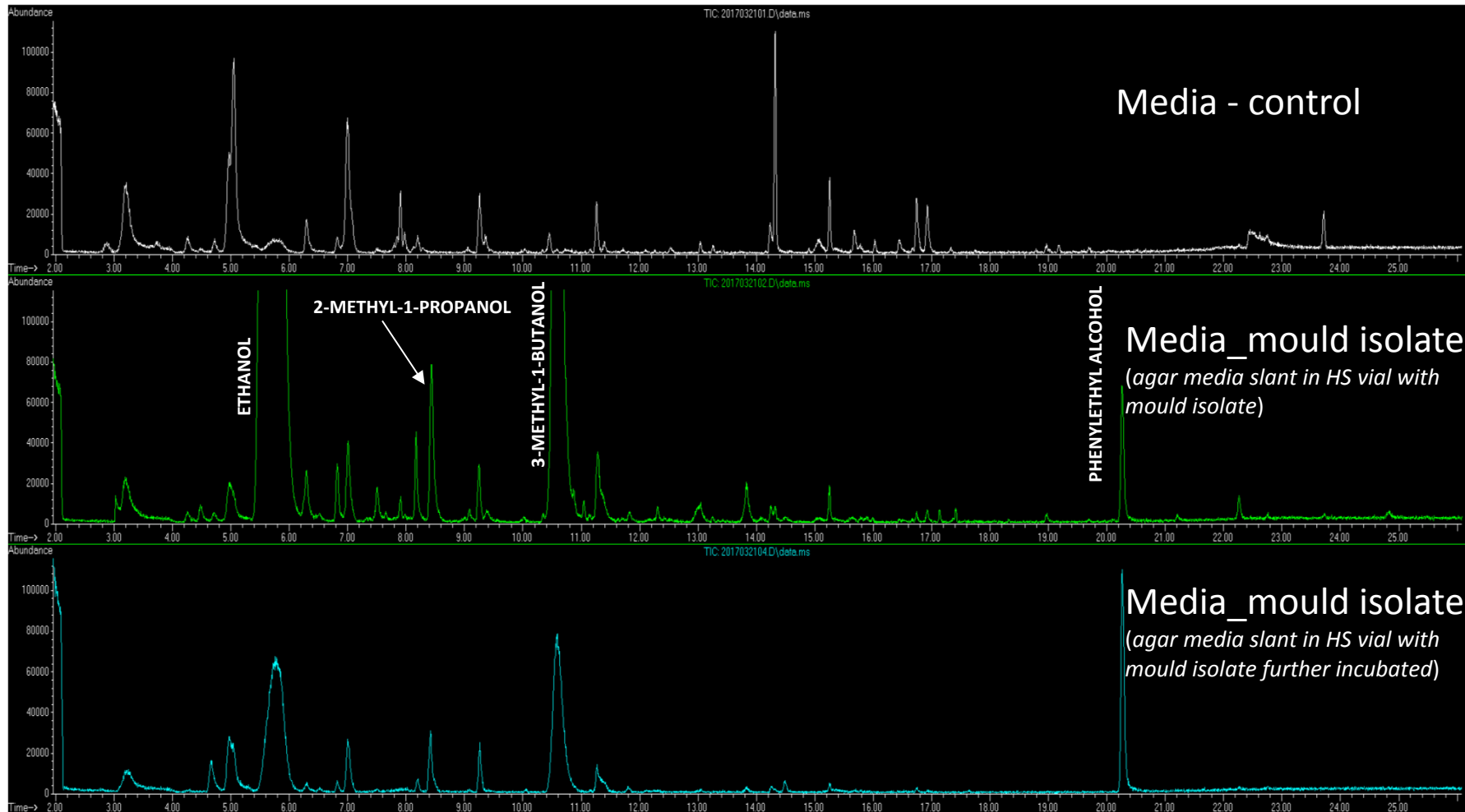


**Fungus isolated from infected burr**



# Non-invasive detection of chestnut rot

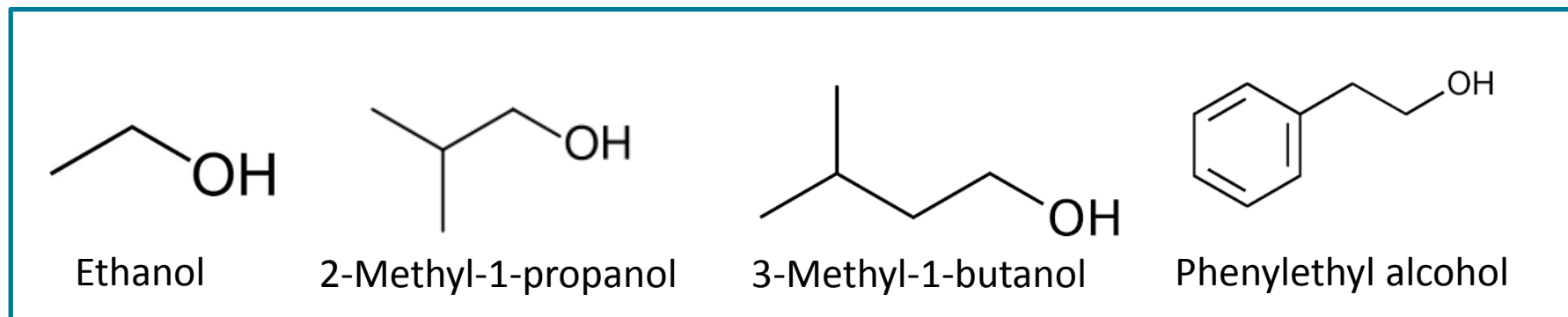
## *Chestnut burr with brown spots*



# Non-invasive detection of chestnut rot

## *Chestnut burr with brown spots*

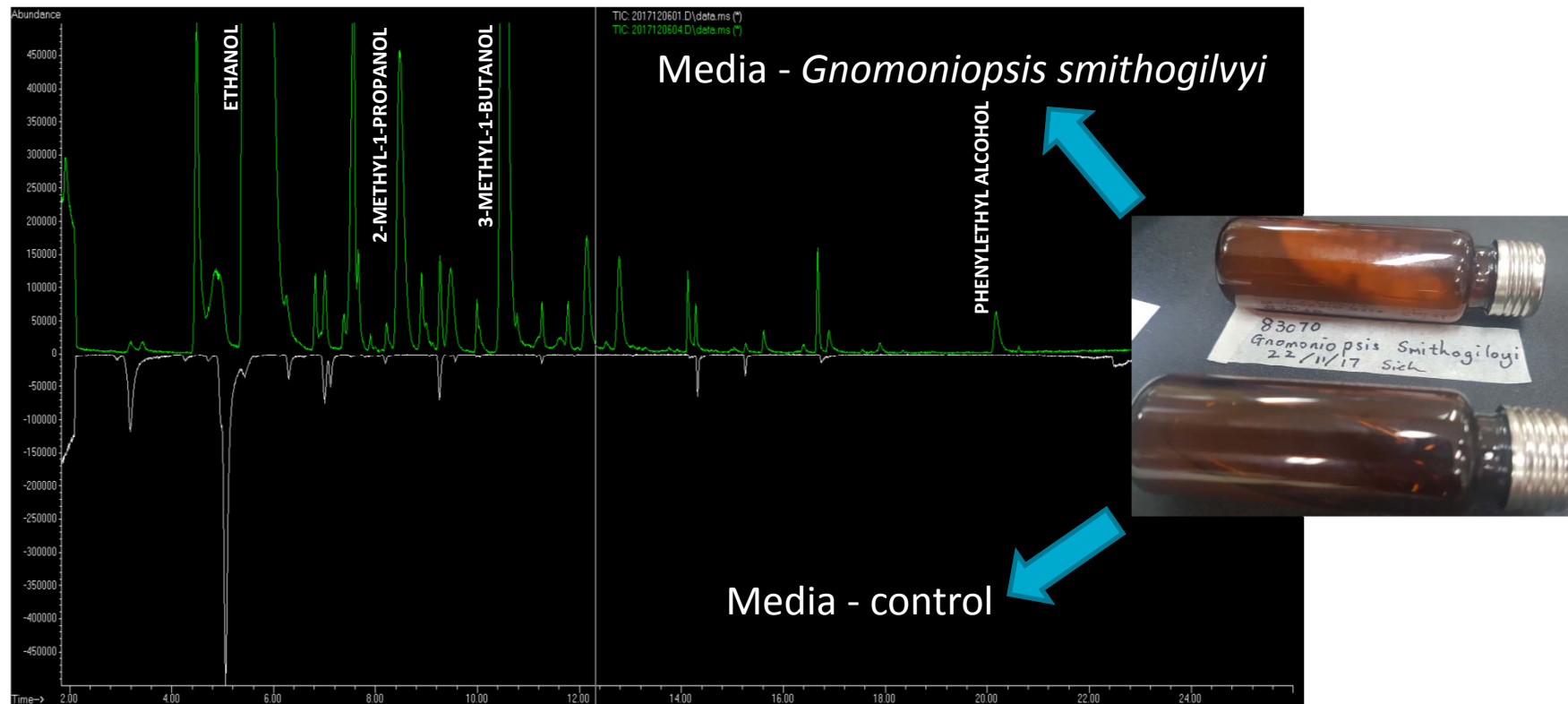
- Mould isolated from chestnut burr samples, showing brown discolouration (collected at an orchard located in Bright (VIC) area) were screened for volatile compounds -
  - As shown in the previous slide – 4 specific volatile organic compounds (VOCs), detailed below, were identified and their origins were clearly attributed to the growth / metabolism of nutrients by isolated mould



- The identity of above mentioned VOCs were compared to compounds produced by *Gnomoniopsis smithogilvyi* culture and further utilized in the non-invasive detection of chestnut rot

# Non-invasive detection of chestnut rot

*VOCs profiling of Gnomoniopsis smithogilvyi on PDA agar*



**FOUR specific VOCs produced by the mould isolated from discoloured chestnut burr were also produced by *Gnomoniopsis smithogilvyi*, responsible for the chestnut rot**



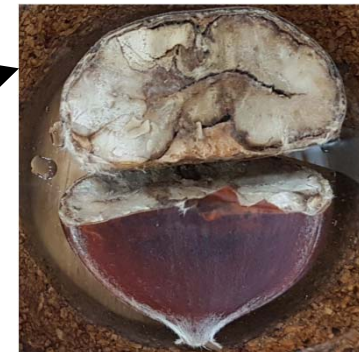
# Chestnut for volatile screening

Purtons and Menzies Chestnut (Stanley, VIC)

Purtons



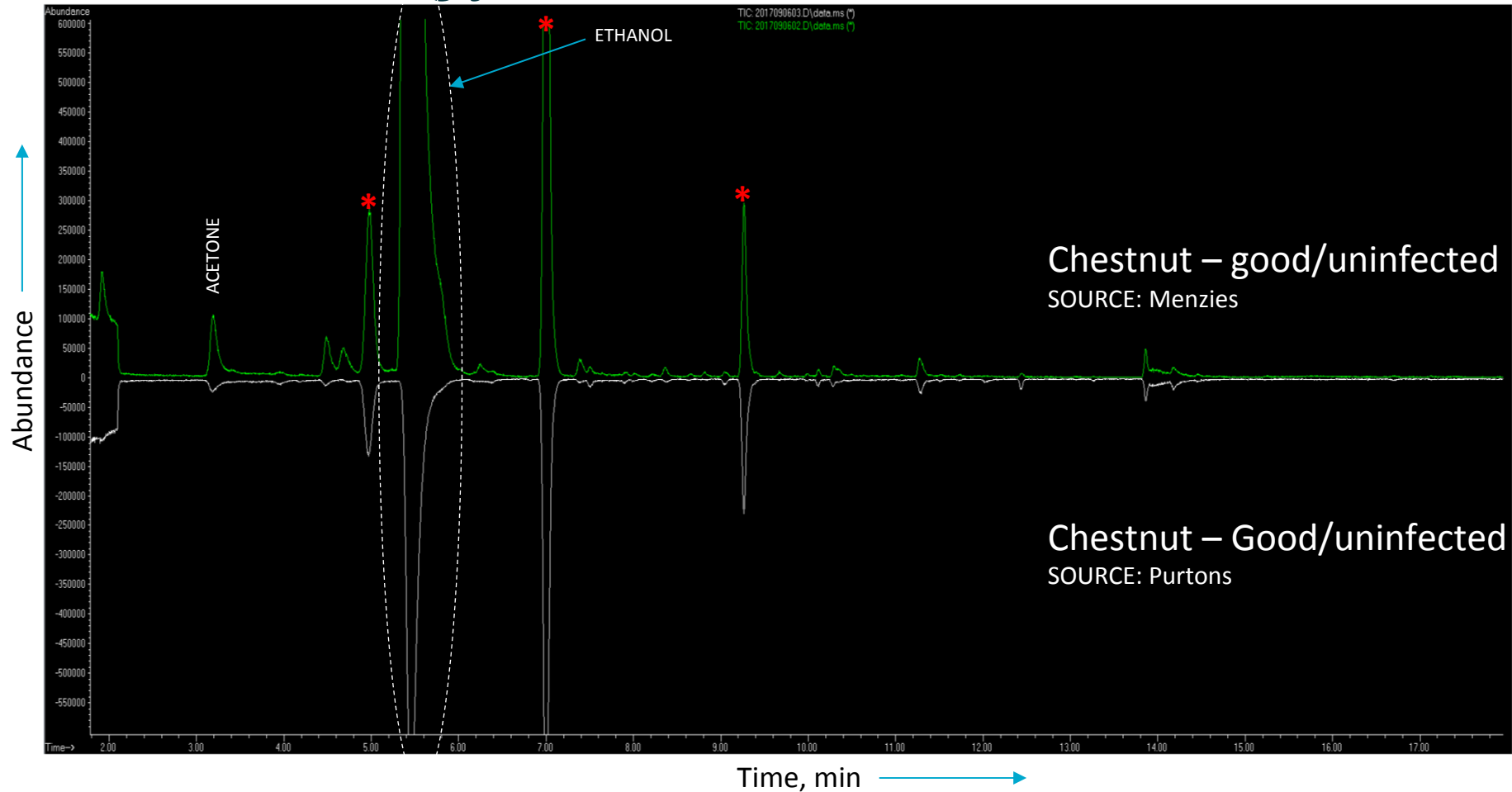
Menzies



One chestnut received (cut into two pieces, held together by *red tape*) clearly identified as infected

# Chestnut

## VOCs emanating from Purtons and Menzies Chestnut



\* Peak did not originate from sample

**VOCs EMANATING FROM FIVE INTACT NUTS**

# Chestnut

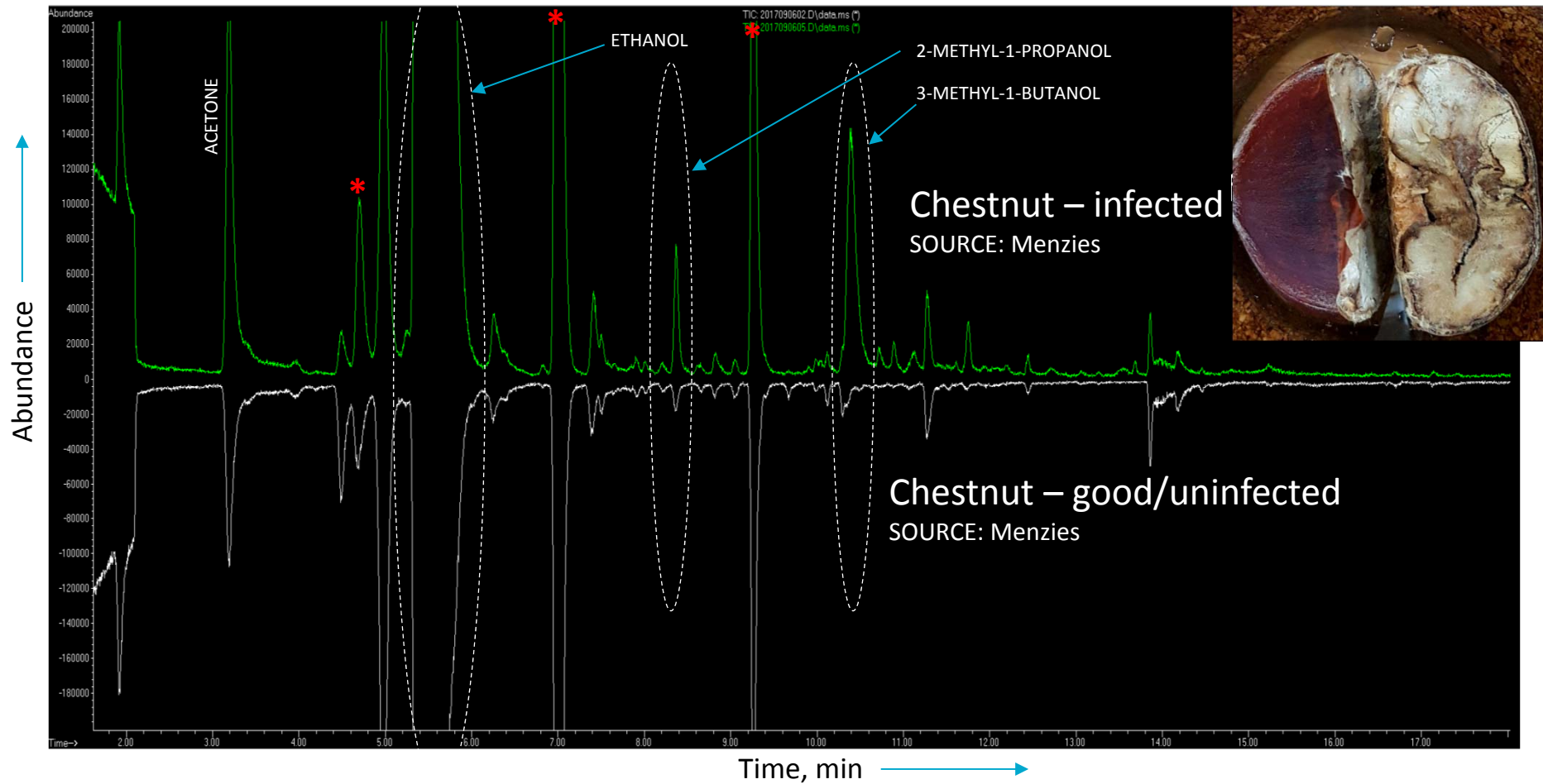
## *VOCs emanating from Purtons and Menzies Chestnut*

VOCs emanating from 5 intact good Purtons and Menzies chestnuts were compared in the previous slide:

- Intact Purtons and Menzies showed remarkably similar VOCs profile
- Both Purtons and Menzies chestnuts only showed presence of ethanol in the profiles and were found to clearly lack the other three VOCs, namely 2-methyl propanol, 3-methyl butanol and phenylethyl alcohol, linked to the chestnut rot fungus
- Both types of chestnuts were found to emanate elevated levels of ethanol which may indicate very early sign of infection

# Chestnut

## VOCs emanating from Menzies Chestnut



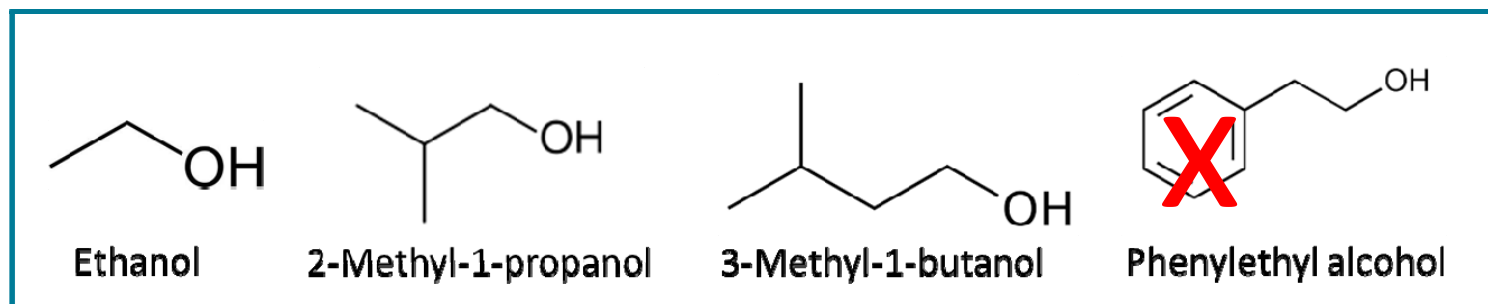
\* Peak did not originate from sample

VOCs EMANATING FROM SINGLE NUT CUT IN HALF

# Chestnut

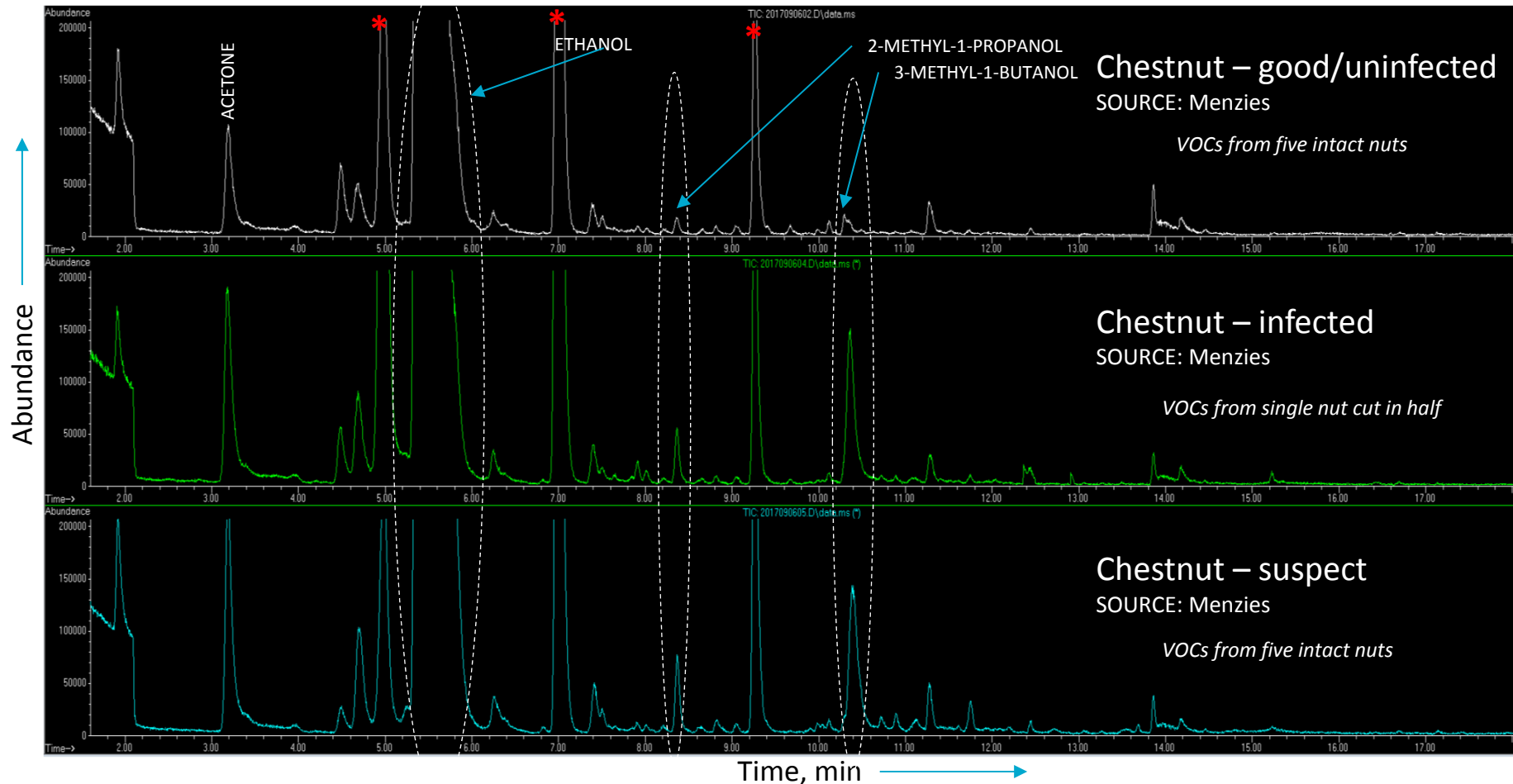
## *VOCs emanating from Menzies Chestnut*

- Comparison of VOCs emanating from uninfected and infected Menzies chestnut (Stanley, VIC):
  - Three of the four biomarkers, namely ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol, of chestnut rot were detected at elevated levels in Menzies chestnut showing clear brown black discolouration



# Chestnut

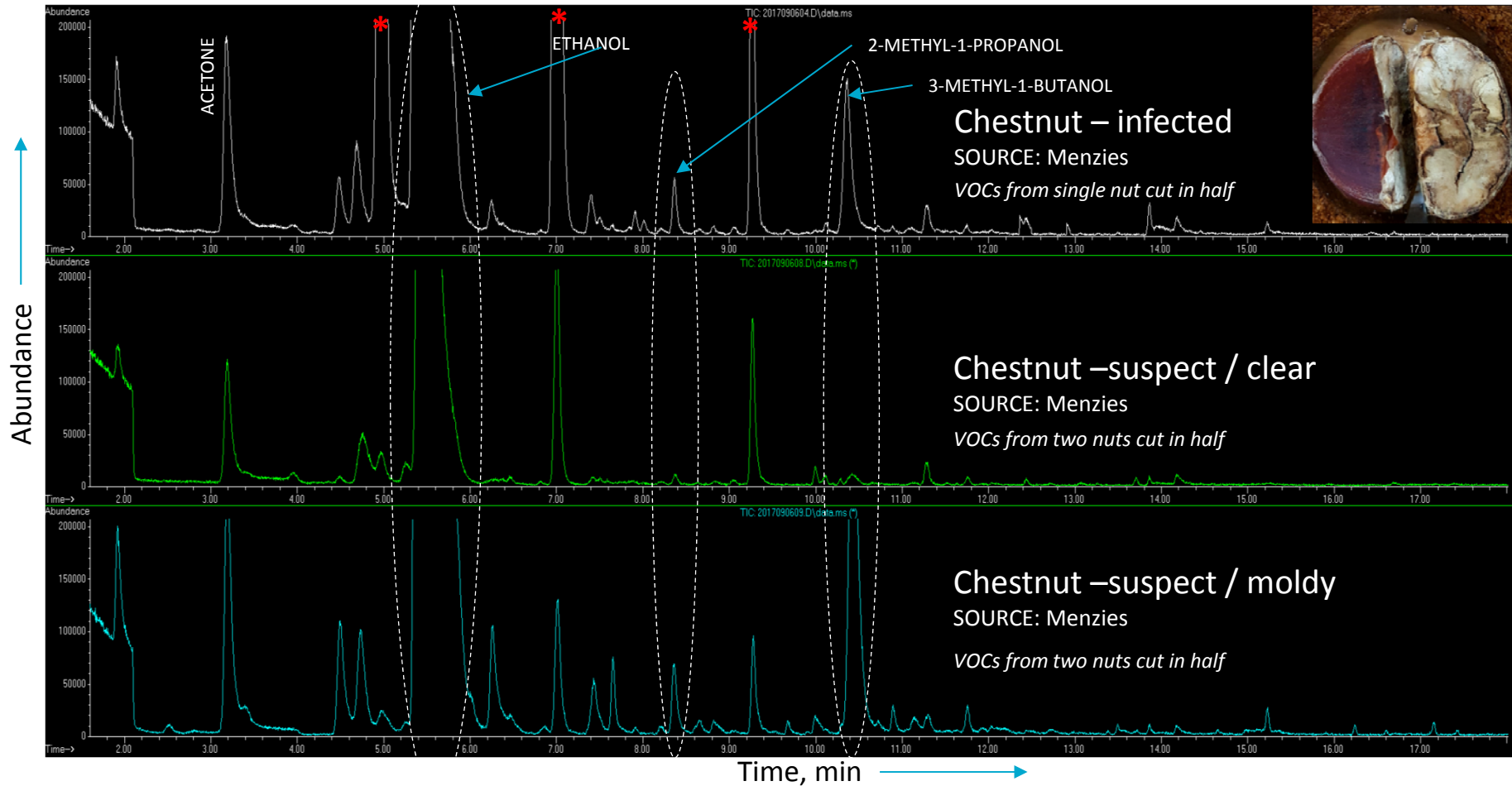
VOCs emanating from suspect, uninfected and infected Menzies chestnuts



\* Peak did not originate from sample

# Chestnut

VOCs emanating from suspect, uninfected and infected Menzies chestnut

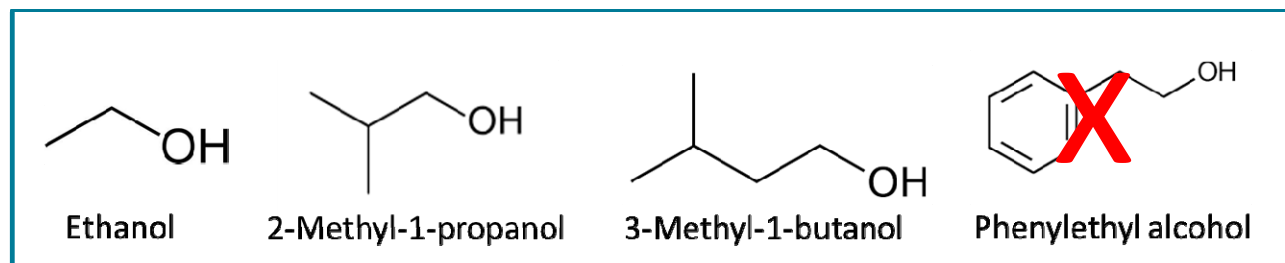


\* Peak did not originate from sample

# Chestnut

## *VOCs emanating from Menzies Chestnut*

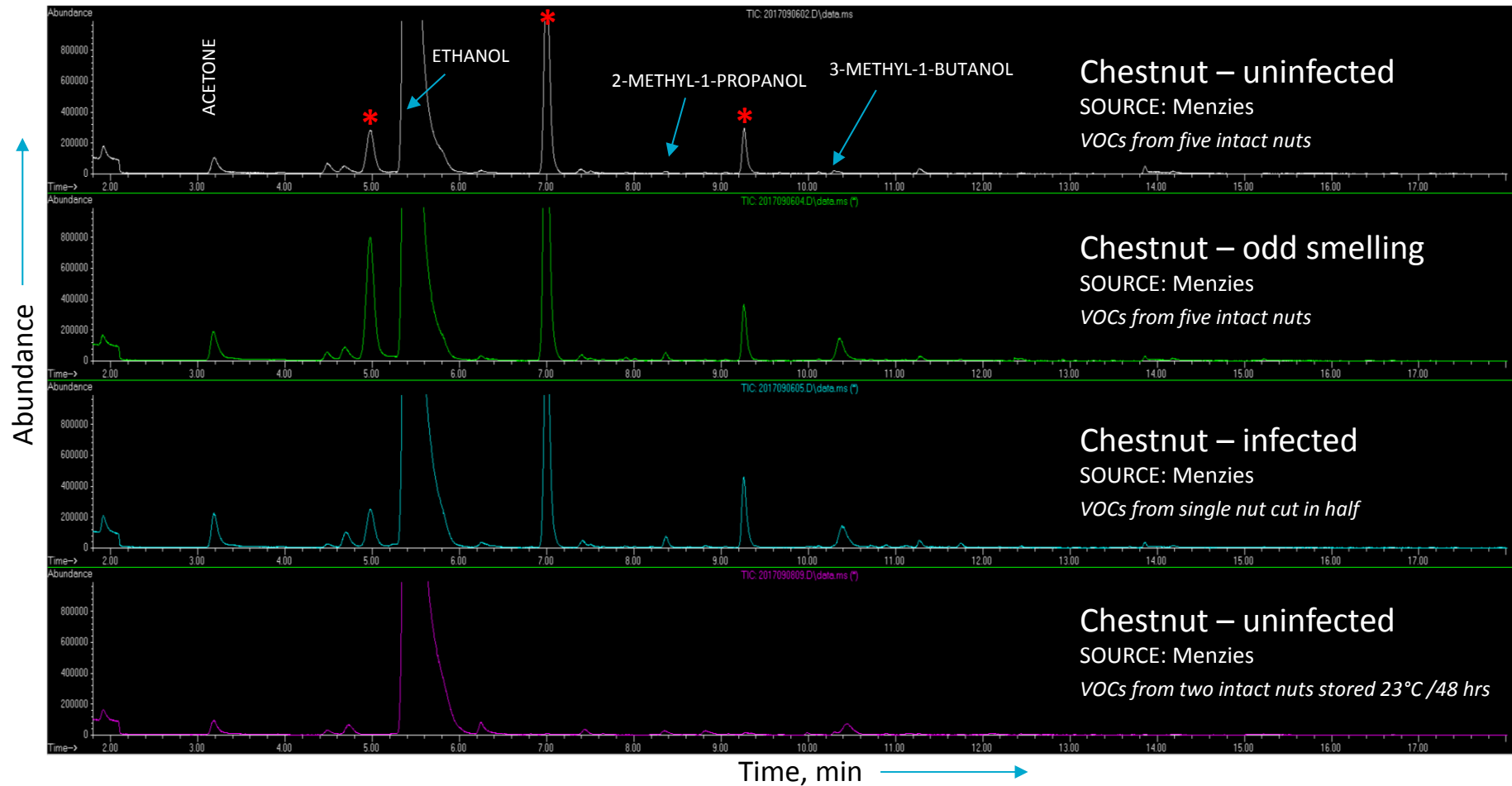
- Comparison of VOCs emanating from uninfected, infected and odd smelling Menzies chestnut (Stanley, VIC):
  - Three of the four biomarkers, namely ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol, of chestnut rot were also detected at elevated levels in odd smelling Menzies chestnut
  - The odd smelling chestnuts were cut in half to observe any sign of discolouration. It was noted that some odd smelling nuts showed discolouration and others were clear. The VOCs emanating from odd smelling clear and discoloured chestnuts were analysed. Discoloured nuts contained above named three biomarkers while the odd smelling clear nuts only had elevated levels of ethanol in the headspace.





# Chestnut

VOCs emanating from suspect, uninfected and infected *Menzies* chestnut

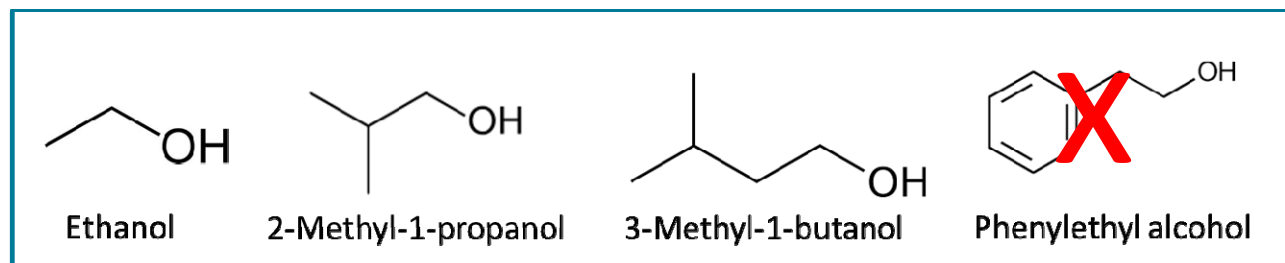


\* Peak did not originate from sample

# Chestnut

## VOCs emanating from Menzies Chestnut

- Comparison of VOCs emanating from uninfected, infected and odd smelling Menzies chestnut (Stanley, VIC):
  - As mentioned earlier, three of the four biomarkers, namely ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol, of chestnut rot were also detected at elevated levels in infected and odd smelling Menzies chestnut
  - Storage of uninfected chestnuts at ambient temperatures (23°C) for 48 hrs lead to slight increase in the production / release of 2-methyl-1-propanol and 3-methyl-1-butanol in addition to ethanol. This may be the early stage development / growth of the chestnut rot causing mould - *Gnomoniopsis smithogilvyi*



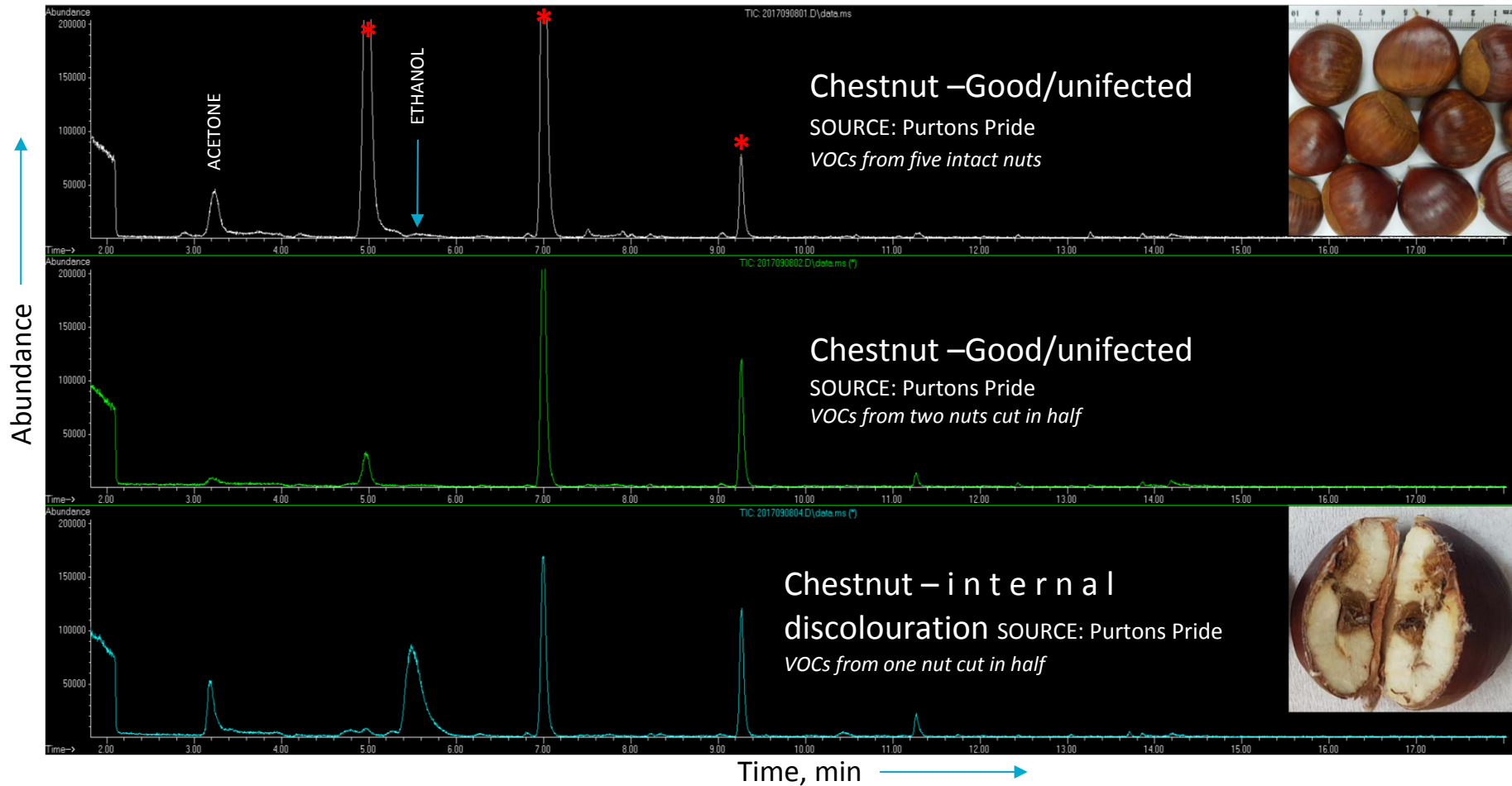
# Chestnut

## *VOCs of chestnuts grown in other regions of VIC and NSW*

Chestnut / burr samples	Details from package / observation	Chestnut Received / Region
Purtons Pride		Sep - 2017; Fumina (VIC)
Easy Peel Me		Sep - 2017; Fumina (VIC)
P Gold L3	stored at room / refrigerated temperature	Sep - 2017; Neerim (VIC)
		Sep - 2017; Tumbarumba (NSW)

# Chestnut

## VOCs emanating from Purtons Pride (Fumina VIC)

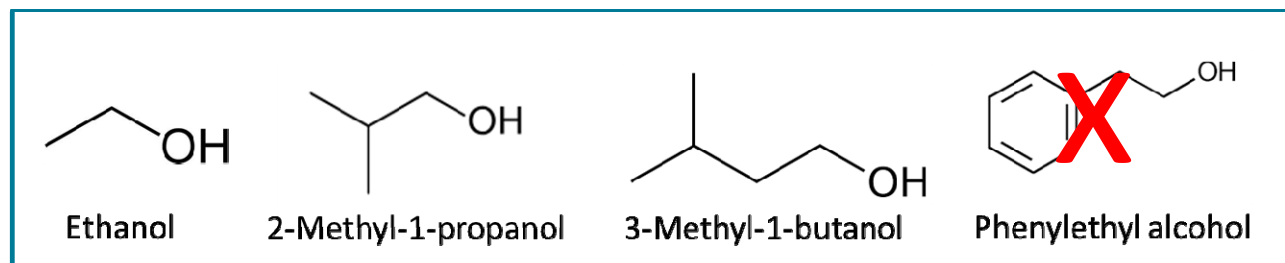


\* Peak did not originate from sample

# Chestnut

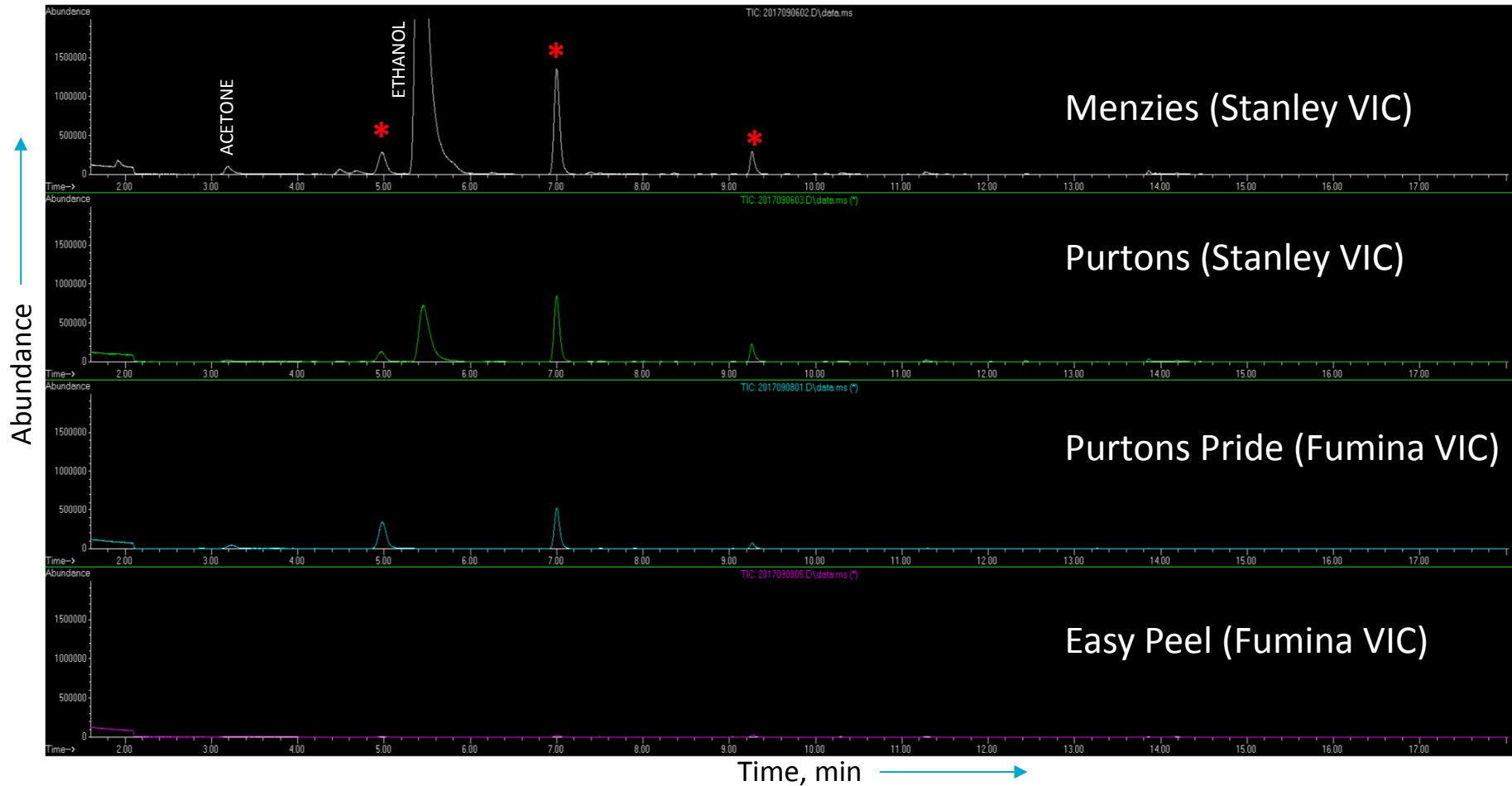
## VOCs emanating from Menzies Chestnut

- Comparison of VOCs emanating from uninfected/good quality and infected Purtons Pride chestnut (Fumina, VIC):
  - Chestnut of good quality / free of infection clearly did not contain any of the chestnut rot biomarker compounds
  - Purtons Pride chestnut clearly showing sign of dark brown patches on internal cut surface (see photo in previous slide) were also found to emit three of the four chestnut rot biomarker compounds- namely ethanol, 2-methyl-1-propanol and 3-methyl-1-butanol. This clearly showed growth /development of chestnut rot mould - *Gnomoniopsis smithogilvyi*



# Chestnut

VOCs emanating from uninfected nuts from different varieties / source

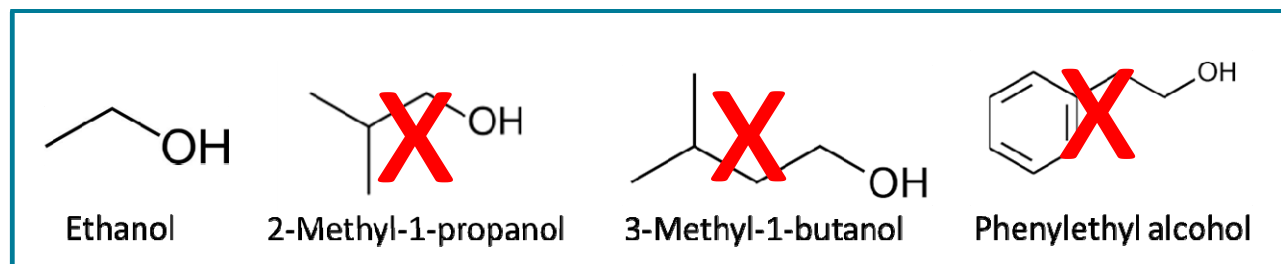


\* Peak did not originate from sample

# Chestnut

*VOCs emanating from chestnuts of different varieties and source*

- Comparison of VOCs emanating from uninfected/good quality:
  - Chestnut of good quality/free of infection clearly did not contain any of the chestnut rot biomarker compounds-
  - Good quality/uninfected Purtons Pride and Easy Peel chestnuts were found to emit none of the chestnut rot biomarker compounds.
  - Uninfected/good quality Menzies and Purtons still contained low levels of ethanol which may indicate very early stages of chestnut rot mould *Gnomoniopsis smithogilvyi* growth/development



# Chestnut

*VOCs emanating from variety “Me” (Neerim VIC) stored at different temperatures on farm*



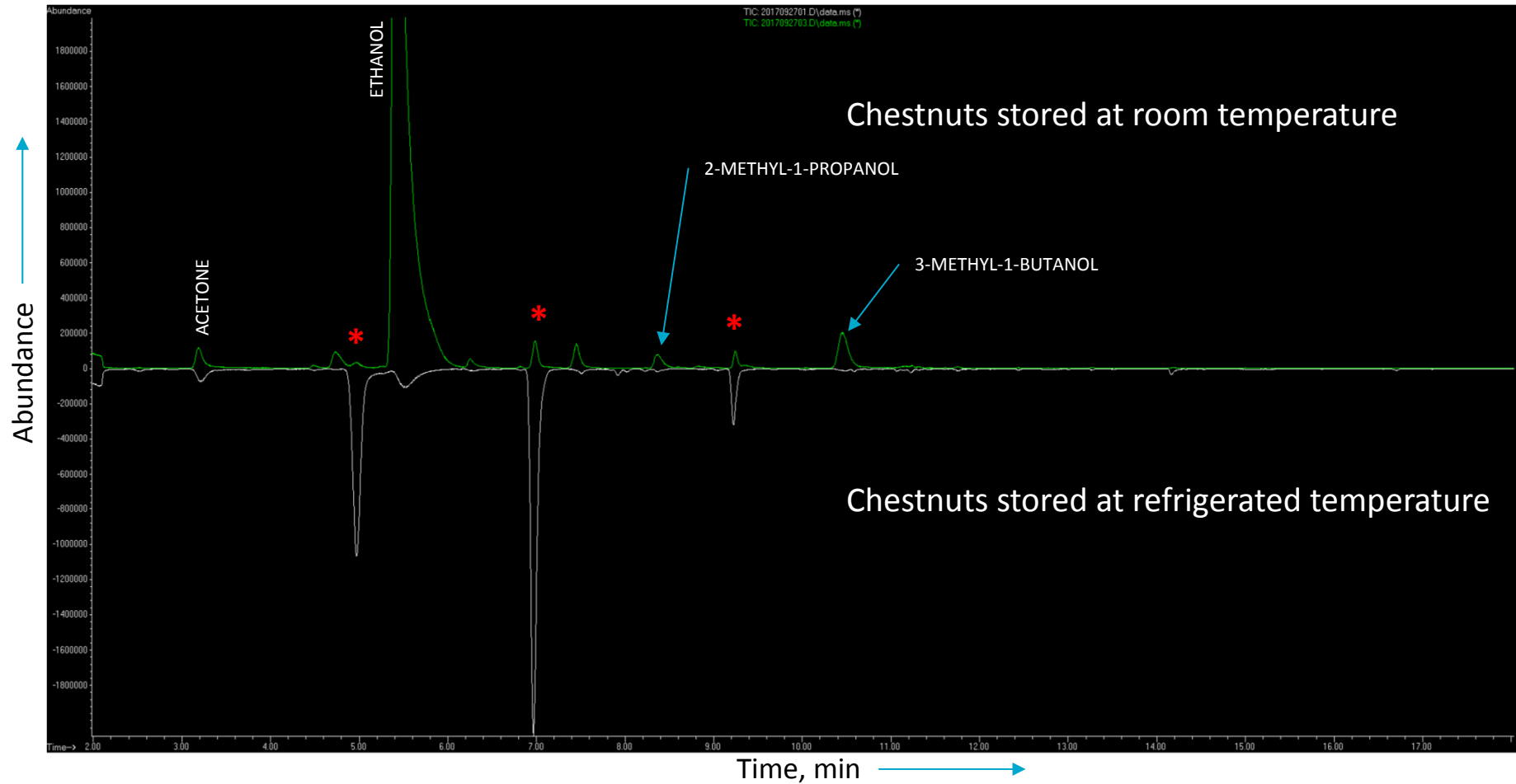
Chestnut stored at refrigerated temperature

Chestnut stored at room temperature



# Chestnut

VOCs emanating from variety "Me" (Neerim VIC) stored at different temperatures on farm

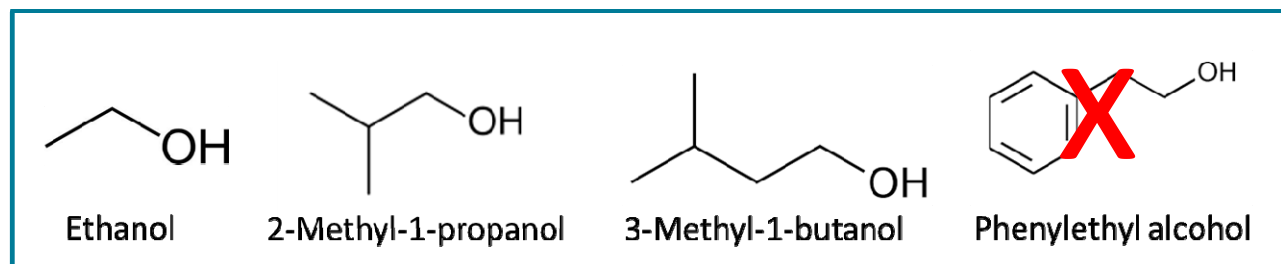


\* Peak did not originate from sample

# Chestnut

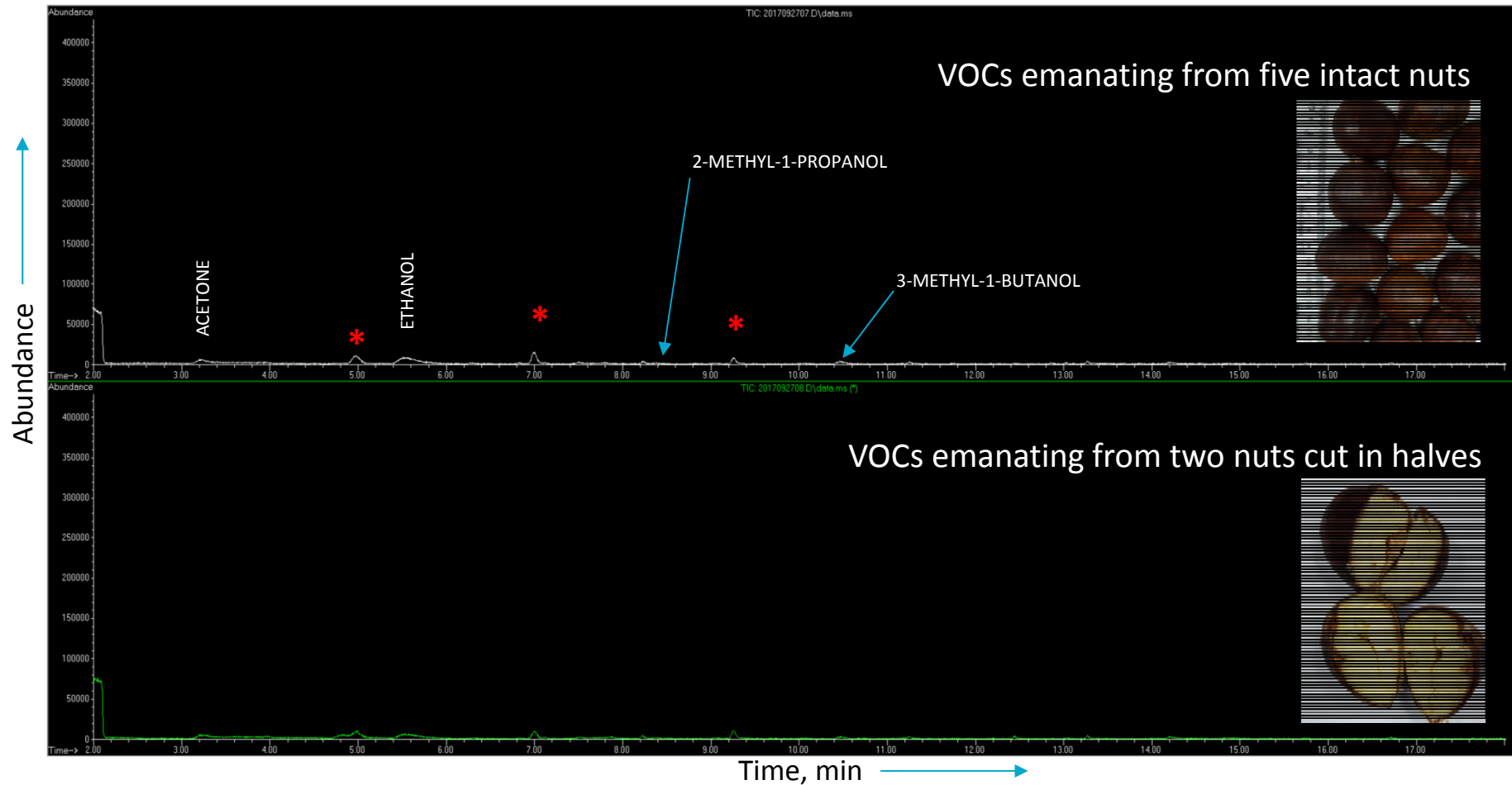
VOCs emanating from variety "Me" (Neerim VIC) stored at different temperatures on farm

- Comparison of VOCs emanating from uninfected/good quality chestnuts stored on farm at different temperatures:
  - Three of the four chestnut rot biomarker compounds were present in the headspace of Chestnuts stored at room/ambient conditions which may indicate early growth / development of mould *Gnomoniopsis smithogilvyi*
  - Nuts stored at refrigerated temperature were found to contain none / trace levels of the chestnut rot biomarker compounds



# Chesnut

## VOCs emanating from variety P Gold (Tumbarumba NSW)

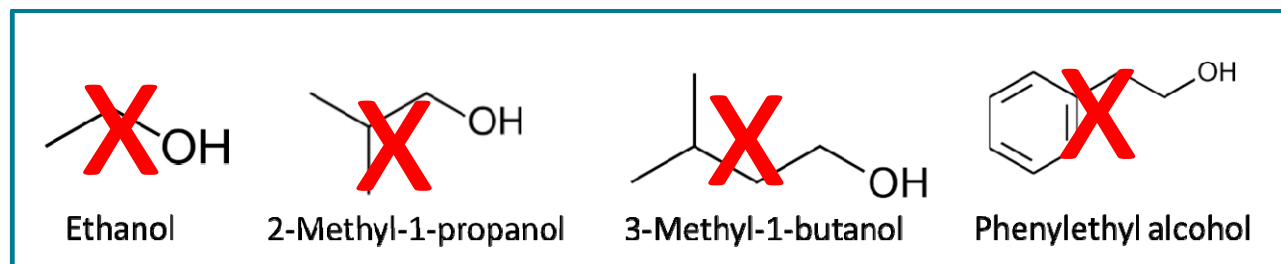


\* Peak did not originate from sample

# Chestnut

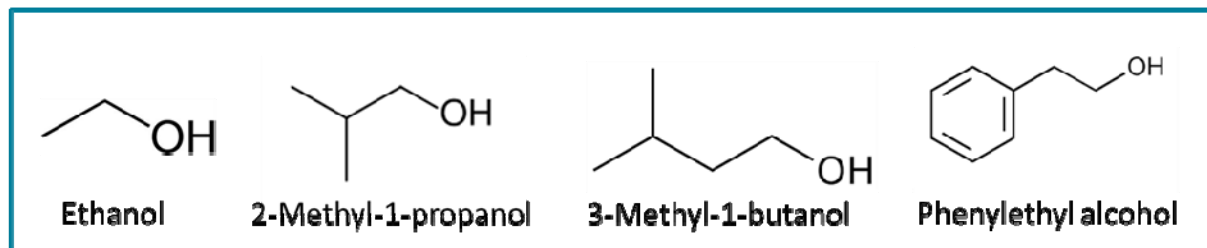
## VOCs emanating from variety P Gold (Tumbarumba NSW)

- Comparison of VOCs emanating from uninfected/good quality chestnuts, intact or cut in half:
  - Chestnut rot biomarker compounds were barely detected in the headspace of P Gold chestnuts (Tumbarumba NSW)
  - *Chestnuts which are uninfected/good quality did emit key chestnut rot biomarker compounds*



# Summary

- Method for the headspace sampling of VOCs was developed. VOCs were sampled using solid phase micro-extraction (SPME) fibre. SPME sampled volatiles were analysed by gas chromatography-mass spectrometry (GCMS)
- VOCs compounds were identified in the headspace over laboratory medium (PDA) inoculated with chestnut rot causing organism *Gnomoniopsis smithogilvyi*. Four biomarker compounds were:



- This observation was further validated by analysing VOCs emanating from infected and uninfected chestnut burrs and chestnuts grown on three different regions of VIC and one region of NSW.
- Chestnuts which were uninfected/good quality did not emit key chestnut rot biomarker compounds named above
- Detection of chestnut rot causing mould through analysis of VOCs is an attractive solution for this problem as it is non-invasive and lends itself to rapid sampling. Sensing technologies, based on biological or electro-chemical approaches, could be screened for their sensitivity and selectivity towards the above named biomarker compounds for the development of a prototype sensor tool for field application
- Further research work is required to determine the most appropriate sensors to develop into prototype sensor tool for field testing.

# Acknowledgements

- Project was supported by funding from Chestnut Australia Inc
- Authors would like to acknowledge technical help of Rangika Weerakkody (CSIRO Agriculture & Food)

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*Farming together.*



# Thank you

**Agriculture & Food**

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