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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- Progress is being made in improving our understanding of the causes of crumbly fruit disorder in raspberry.

Background and expected deliverables

In defining a strategy for studying and resolving the problem of crumbly raspberry fruits, we must first ask; what is the crumbly fruit condition?

Little scientific literature is available on this specific topic and above all no standard definition is available. In a previous published paper '*Towards an understanding of the control of crumbly fruit in red raspberry*' (Graham *et al*., 2015), the authors refer to it as a condition linked with pollen abortion and embryo sac degeneration causing drupelets to be generally reduced in number but greatly enlarged or, in the case of small reductions, cohere imperfectly so the fruit crumbles when it is picked. To solve or understand this problem, every aspect related to it is important. An understanding of the flower and fruit anatomy as well as of the processes leading to fruit set and developments is essential.

Raspberry fruits are formed from an aggregation of multiple fertilized ovaries each of which is referred to as a drupelet as it becomes fleshy. It is thought that crumbly fruit is caused by a partial failure in one or more physiological processes concerned with fruit development, which results in the drupelets not forming properly.

This is an increasing problem for the global raspberry industry. There have been a number of causes suggested for the condition. It is known that infection with certain viruses can increase the likelihood that fruits become crumbly (Jennings, 1988). Raspberry Bushy Dwarf Virus (RBDV) affects pollen reducing its capacity to induce fruit-set and can lead to failure of almost half of all drupelets to set (Murant *et al.,* 1974; Daubeny *et al.,* 1978).

A genetic cause has also been demonstrated where the crumbly phenotype arises from virus-tested mother plants (Jennings, 1988). Studies have also shown that extensive tissue culturing of plants may increase the emergence of the condition (Jennings, 1988). Additionally, environmental conditions appear to play an important role with variations in the extent of crumbliness apparent from year to year (A. Dolan *pers. comm.*).

Although no standard definition is available, a common interpretation of the phenomenon is required to address the issue and find a solution. For such reasons, two new definitions of crumbly fruit have been proposed:

Crumbly Fruit Condition (**CFC**) only affecting plants where all the fruits are symptomatic year after year; this is an undefined disease or genetic disorder.

Malformed Fruit Disorder (**MFD**) only involving plants displaying malformed fruit that resemble crumbly fruit in their appearance but where the symptoms are intermittent within a year or over the subsequent years. **MFD** plants display uneven fruit set, generally at the very beginning of fruit production and this occurs mainly on the top lateral shoots. Fewer symptoms are observed as the season progresses and more flowers and fruits are produced by the plant resulting in little or no loss of yield.

These new definitions will be included in an online survey that will be launched in late autumn 2017 to gather information from growers on the varieties grown, location of the plantations, **CFC** and/or **MFD** affected plants, origin of plants and agronomic procedures used. The results will help to estimate the spread of crumbly fruit.

The practical research in this Ph.D. studentship project intends to define the trigger(s) of the condition by inducing crumbly fruit on raspberry plants grown both in optimal conditions (i.e. growth room and glasshouse) and sub-optimal conditions (i.e. field). The induction of crumbly fruit will allow the study of the genetic and physiological control behind this condition. The project will then be divided into three main work activities:

1. crumbly fruit induction
2. hormones profiling
3. gene expression.

Understanding these factors will help to identify genes linked to crumbly disorder which in turn could provide tools to help the selection of new varieties which are free and/or resistant to the disorder. Alternatively, hormonal/chemical treatments could be developed to be used on plants during their growth phase to avoid crumbly fruit.

Summary of the project and main conclusions

Early work in the first year of this Ph.D. project has induced crumbly fruit in a growth chamber by removing the carpels of the flower. This has demonstrated that their removal and/or

wounding of the receptacle can trigger crumbly fruit in raspberry plants. Data from both glasshouse and field experiments seem very promising too. At the time of writing, data is still being analysed.

Financial benefits

According to Defra's horticultural statistics in 2013, the UK produced 13,800 tonnes of raspberries which were worth £89.6 million. It is estimated that a further 10% of fruit may have been marketed had it not been discarded due to crumbly fruit disorder and this would have increased the total to 15,180 tonnes, representing an extra £9 million.

The study and comprehension of the physiological and genetic causes behind crumbly fruit represent a starting point for developing a new test method for the condition which is faster and more reliable than the current fruiting test. The molecular knowledge gained from this project could be utilised in the UK Raspberry Breeding Programme to select new varieties which are free from the disorder or at least more resistant to this condition. Developing varieties which are free from the disorder could therefore increase the value of the UK crop by £9 million per annum.

Action points for growers

- At this stage of the project, no action points for growers have been identified.

SCIENCE SECTION

Introduction

Raspberries belong to the genus *Rubus* in the *Rosaceae* family. This comprises a highly number of species, 250-700 depending on classification (Thompson, 1997). The genomic number is 7 and ploidy levels range from diploid to dodecaploid (Jennings, 1988; Meng and Finn, 1999). Members can be difficult to classify into distinct species due to hybridization and apomixes (Robertson 1974), but molecular studies of phylogeny (Alice and Campbell, 1999; Sochor *et al*., 2015; Wang *et al*., 2016) are assisting the development of a robust phylogeny for *Rubus*. Species are distributed widely across Asia, Europe, North and South America, with the centre of diversity now considered to be in China. For a brief history of the crop from ancient times see Kellogg *et al*., (2011).

Details of the growth cycle have been described in detail by Jennings (1988). Briefly, raspberries are woody perennials with a biennial cane habit, though primocane varieties are increasingly important commercially. Small flowers are initiated in the second year of planting and these have five sepals, five petals, a very short hypanthium, many stamens, and an apocarpous gynoecium of many carpels on a cone-like receptacle. Multiple ovaries each develop into a drupelet and the aggregate fruits are composed of the individual drupelets held together by almost invisible hairs. Fruiting begins in the second year of planting and in favorable conditions plantations can continue to fruit for more than 15 years. In raspberries day length and temperature cause shoot elongation to cease and leaves form a terminal rosette at the shoot tip and dormancy sets in gradually. Raspberries represent a valuable horticultural commodity, both as a source of income and also labour, as most fruit for the high-value fresh market is hand-harvested.

Most raspberry production is concentrated in the northern and central European countries, although there is an increasing interest in growing cane fruits in the southern Europe e.g. in Greece, Italy, Portugal and Spain. In many production areas, the fruit is grown for the fresh market, but in central Europe e.g. Poland, Hungary and Serbia, a high proportion of the crop is destined for processing. Major regions of production in North America include the Pacific North West, California, Texas and Arkansas, as well as regions in New York, Michigan, Pennsylvania and Ohio. Chile, Argentina and Guatemala also have extensive production. Increasing popularity of autumn-fruiting raspberries, in which late season fruit is harvested from berries forming on the upper nodes of primocanes (Jennings and Brennan, 2002), has extended the production season and the period of attack of some foliar and cane pests. Some very early spring fruits with high value can also be obtained from the remaining lower nodes of these over-wintered primocane-fruiting types. Primocane-fruiting raspberries tend

to be grown in the warmer areas of Europe where the temperature in autumn is relatively high and there is little risk of early autumn frosts. Interest has also been shown in extended-season production under glass or under plastic structures in the northern Europe countries, e.g. Belgium (Meesters and Pitsioudis, 1999) and the UK and now in the Mediterranean fringe e.g. Spain and Greece and this trend will affect their pest and disease status. To satisfy these production systems, long primocanes grown in northern regions, such as Scotland, are lifted, chilled and stored for long periods for planting in late spring for late summer harvest under plastic. The concept of extended-season-production has been so successful that by careful manipulation of plant dormancy cycle and flower initiation it is now possible to produce fresh raspberries in Europe for sale in almost all months.

The crops are also produced for processing markets, including high value berry juices for their flavour and perceived health benefits. Demand for these berry crops is at an all-time high with production rapidly increasing (Strik *et al*. 2007), mainly fuelled by perceived health benefits. The fact that *Rubus* berries have some of the highest levels of antioxidants and phytonutrients of any fruit crop, due primarily to their intense concentration of anthocyanins and phenolic compounds (Moyer *et al*., 2002) has led to a number of investigations on antioxidant levels of raspberries (Anttonem and Karjalainen 2005; Beekwilder *et al*. 2005; Dossett et al. 2008; Moore et al. 2008; Weber et al. 2008).

Fruit development in raspberry

Raspberry fruits are formed from an aggregation of multiple fertilized ovaries each of which are referred to as drupelets once they become fleshy. A condition known as crumbly fruit occurs to differing degrees in different raspberry varieties and is an indication of partial failure in the physiological processes in fruit development. There have been a number of causes suggested for the crumbly condition:

- Virus infections (Quito-Avila et al., 2014) can increase the likelihood that plants become crumbly, i.e. Raspberry Bushy Dwarf Virus (RBDV) infects the pollen reducing its capacity to induce fruit-set;
- Extensive tissue culturing of plants may increase the emergence of the condition (Jennings, 1988);
- Environmental factors such as low or high temperatures at particular time points in development.

Some cultivars appear to be more prone to the condition than others but in cases where the environmental conditions differed from the normal seasonal level then random symptoms of crumbliness could be displayed in cultivars not previously known for the problem.

The genetic basis of raspberry fruit development is not well understood, although some studies have been carried out to look at overall control of fruit development and aspects of ripening: McCallum *et al*., 2013; Paterson *et al*., 2013; Dobson *et al*., 2012; McCallum *et al*., 2010; Graham *et al*., 2009; Simpson *et al*., 2016. Recently some progress has been made in determining that both genetic and environmental factors affect the crumbly phenotype. A location on Linkage Group 1 (LG1), highly significant for determination of the “crumbly fruit” syndrome, in red raspberry, has been identified and it is robust across seasons and in different environment (Graham *et al*., 2015).

The current model of regular fruit set implies that ovary growth is blocked before pollination and that auxin is a key regulator of ovary growth de-repression at fruit set; other phytohormones have a role in fruit initiation and development, i.e. gibberellin, cytokinin, brassinosteroids, ethylene and abscisic acid; (Goetz *et al*., 2007; Pandolfini *et al*., 2007).

Fleshy fruits are botanically diverse in the way they develop, whereas raspberry, tomato and grape are derived from the ovary, other fruits such as apple and strawberry are derived from the receptacle or from the expansion of the sepals. Despite these botanically differences, all the fruits undergo a similar development steps including: fruit set, growth, maturation and ripening. Fruit set is the first stage of the development after the fertilisation event, it is followed by an active cell division and expansion phase, called growth, that phase causes the fruit to attain its maximum size. This is followed by a stage where the fruit acquire the prerequisite competence to enter in the final developmental stage, i.e. ripening. Fleshy fruits are physiologically classified as climacteric and non-climacteric with the first showing concomitant increase of respiration and ethylene biosynthesis upon initiation of ripening while the non-climacteric lacking these two attributes at the onset of ripening (Kumar *et al*., 2014).

The combined action of three hormones: auxin, gibberellins (GA_s) and cytokinin plays a major role in the regulation of fruit set. Importantly, auxin, GA and cytokinin levels increase at fruit set and the requirement of their higher levels at fruit set has been already validated by their exogenous treatment, which causes parthenocarpic fruit formation in tomato; see Kumar *et al*., (2014); the authors suggest that auxin promotes fruit set and growth, at least partially, by controlling the GA levels. Differential regulation of many genes related to biosynthesis and signalling of other phytohormones, including: ethylene, abscisic acid (ABA), cytokinin and brassinosteroids (BR) further suggested that besides auxin and GA, these hormones are also important during fruit set and early fruit development stages. From a molecular point of view, the fruit set involves the concerted action of auxin and/or GA and/or cytokinin or BR through the biosynthesis and/or the signalling which regulates the activation of core cell cycle genes

during early fruit development stages; ABA and ethylene play antagonistic role in fruit set even though such mechanisms remain unidentified. Seed and fruit development are intimately connected and synchronized, seeds are rich sources of hormones, auxins, GA and cytokinin above all, which are involved in stimulating the growth of the surrounding tissues and even in determining the fruit size. Interplay between these hormones is necessary for fruit growth and, so far, the established role of auxin, in the regulation of cell expansion, seems to be the most important during this development phase. Once the cell division stage is over, auxin and GA become the main regulators of the cell expansion phase. These hormones are localized mostly in the seeds and then transported to the surrounding tissues but, except for the auxin, the knowledge on this aspect remains limited.

In Pomares-Viciana *et al*., (2017), the role of auxin in regulating fruit development after pollination, is even more explicitly highlighted, in fact the authors, studying the parthenocarpic development process in zucchini, have found how fruit development is closely related to auxin response genes such as: ARFs (Auxin Response Factors), Aux/IAAs (indole acetic acid) and TIRs (TOLL/Interleukin-1 receptors) encoded by multigene families. Transcriptome analyses of *CpARF8, CpIAA9* and *CpTIR1* have revealed that they show tissue-specific expression, maintaining the structure and function showed in other species. Such key auxin signalling genes showed a specific level of quantified mRNA in pre-anthesis and anthesis that changed after the fertilisation cue, supporting their role in the preparation of the ovary to become fruit in zucchini. A similar scenario could be found in raspberry where, among other things, the potential development of parthenocarpic drupelets cannot be completely excluded.

Raspberry fruit is complex, it is an aggregation of multiple fertilized ovaries referred to as drupelets once they get fleshy and juicy, on average a berry consists of around 75-85 drupelets. Since a raspberry flower consists of 100-125 carpels, to get a proper fruit, on average, at least 3/4 of carpels must be fertilized (Fuentes *et al*., 2015; Jennings, 1988).

Many hypotheses can be proposed for the triggers of the crumbly condition in raspberry; some of the main factors that may be responsible for causing the crumbly condition in raspberry are:

- Failed pollination: i.e. too few ovaries fertilized, keep in mind that at least 75-85 ovules, out of 100-125, must be fertilized to get a proper berry;
- Damaged flowers: i.e. each flower must be visited by the pollinators just four times, over-visiting can easily and fatally compromise fruit development (see Saez *et al*., 2014);
- Adverse environment conditions during key stages of flower/fruit development: i.e. temperatures too high and/or too low;

— Plant pathogens: i.e. viruses above all; see Quito-Avila *et al*., 2014.

Each of these stresses (abiotic and biotic), alone or in combination, are responsible for the activation of signalling pathways which involve intermediates and crosstalk through: hormonal, redox and enzymatic components. Hormones are key players in the plant response in case of stress condition but are even directly involved in the normal development of fruits (Kumar *et al*., 2014). In general: auxins, gibberellins and cytokinins, interacting together or alone, play a crucial role in the first two phases of fruit development namely, fruit set and fruit growth. These are the stages when the crumbly condition appears. Understanding the hormones involved in fruit set together with those activated during stress conditions, in crumbly and non-crumbly fruit is a key factor to better understand the trigger(s) of such condition and possible find solutions to monitor and then avoid it.

According to data available in literature, in raspberry there is a systematic and consistent increase in drupelets number when plants are developed in short day (SD) and low temperature (LT) conditions during flower induction (Woznicki *et al*., 2016); in fact female sex expression is generally favoured by SD and LT and is often linked to the strength of photoperiodic induction.

Flowering is regulated by a range of environmental and physiological signals (Fornara *et al*., 2010; Song *et al*., 2012) that need to be fully understood in perennial crops. Activity of CONSTANS (CO) a key component in leaves of the photoperiodic pathway accumulates under long day conditions and activates transcription of FLOWERING LOCUS T (FT) (Simon *et al*., 2015) which interacts with bZIP transcription factors (Abe *et al*., 2005; Cao *et al*., 2016) activating a cascade of downstream genes leading to flowering. This basic flowering process is impacted by a number of autonomous and stress related signals. For example, the MADS box FLOWERING LOCUS C (FLC) and short vegetative phase proteins (SVP) form a complex to repress flowering until the plant is exposed to the appropriate level of cold. In raspberry, RiMADS_01 was identified as a potential candidate affecting vernalisation (Graham *et al*., 2009a).

These environmental effects are responsible, at molecular level, for changes in the endogenous plant hormones levels with auxin favouring female and gibberellins male sex expression. Increased femaleness in the form of a boosting of carpels (pistils) per flower is a characteristic also in *Rubus* species with perfect (hermaphrodite) flowers. Crumbly condition could be the result of plants having disproportionate rate pistils/stamens. It seems possible that a very low number of pistils per flower (decreased femaleness), paired or not with a high number of stamens, could be the easiest scenario responsible for crumbly condition. As long as not enough ovaries get fertilized, the fruit can be misshapen. Together with hormones, other molecules could participate in crumbly condition to happen, and within these there are

ROS (Reactive Oxygen Species), mainly superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), both are sub-products of cell respiration and are even plant stress response signals (Huan C. *et al*., 2016). The study and the monitoring of their content in: receptacle, fertilized ovaries, early stage fruit setting and ripen fruits in both crumbly and non-crumbly plants would give as a more detailed overview of the situation in terms of:

- Activated molecules during this condition;
- Their amount;
- Role played;
- Phase/stage of the fruit growing when such molecules are involved.

Materials and methods

RNA extraction (red and green berries)

In preparation for extraction, pestles, mortars and glassware were wiped with RNase ZAP wipes (ThermoFisher Scientific, USA) then rinsed with a 0.1% (v:v) solution of diethyl Pyrocarbonate (DEPC) (Sigma-Aldrich, USA) and finally autoclaved (120°C, 15 psi for 20 minutes). 50 ml centrifuge tubes (ThermoFisher Scientific, USA) were soaked in a 0.1% (v:v) solution of DEPC, incubated at room temperature for approximately 18 hours and autoclaved (120°C, 15 psi for 20 minutes). Solutions were treated with 0.1% (v:v) DEPC for approximately 18 hours at room temperature and autoclaved. For all RNA handling methods, RNAse free sterile pipette tips and Eppendorf tubes (Eppendorf, Germany) were used. For all manipulation of RNA, a nuclease-free environment was maintained. Gloves were always worn and changed frequently to prevent RNase contamination.

Four grams (red berries) and two grams (green berries) fresh weight of frozen fruit was used for each RNA extraction. Samples were ground to a fine powder in liquid nitrogen in a cooled pestle and mortar and allowed to stand briefly at room temperature while excess liquid nitrogen volatised. 20 ml of a 1:1 (v:v) solution of lysis buffer (2% SDS, 50 mM EDTA, 300 mM Tris), adjusted to ph 8.0 with boric acid, and phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10mM Tris ph 8.0, 1 mM EDTA (Sigma-Aldrich, USA) pre-heated to 80°C, was added. The mixture was transferred to a centrifuge tube and insoluble polyvinylpolypyrrolidone (PVPP) was added to a final concentration of 8.5% (w:v). The mixture was vortexed for one minute and then centrifuged at 20,000 g for 10 minutes at 4°C. Approximately 13 ml of the upper aqueous phase was removed to a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) added. The mixture was vortexed and

centrifuged as above. Approximately 12 ml of the upper aqueous phase was removed to a fresh tube and 1/3 volume of 12 M LiCl (Sigma-Aldrich, USA) was added, mixed thoroughly and incubated at 4°C overnight. The tube was marked to indicate the expected area of the RNA pellet and centrifuged at 20,000 g for 90 minutes, at 4 °C. The supernatant was decanted and the pellet was gently washed with 5 ml ice-cold 70% (v:v) ethanol and then the alcohol was decanted from the tube which was then inverted to dry the pellet. The RNA pellet was resuspended in 1 ml of DEPC treated sterile water and transferred to a two ml Eppendorf tube. Following centrifugation at 8,000 g for 5 minutes at 4°C to remove any insoluble material, the supernatant was transferred to a fresh 2 ml Eppendorf tube. LiCl was added to a concentration of 0.8 M followed by 1 ml of propan-2-ol. The mixture was incubated AT -20°C for at least two hours. Then, the mixture was centrifuged sat 16,000 g in a bench top centrifuge (Heraeus FRESCO 17, Thermo-Scientific, USA) for 30 minutes at 4°C to pellet the RNA which was then washed with 1 ml ice-cold 70% ethanol by dislodging and pipetting over the pellet approximately ten times. The Eppendorf tube was centrifuged briefly and the ethanol decanted. Any residual ethanol was removed by incubating the inverted Eppendorf tube at room temperature for 5 minutes. The RNA pellet was resuspended in 50 to 100 µl of DEPC treated sterile distilled water. For the quantitative determination of RNA, an instrument detecting the UV-Visible light absorbance method was used (NanoDrop™, ThermoFisher Scientific, USA). The RNA was stored, frozen at -80°C until required.

RNA extraction from open flower buds (OFP)

The same procedure as above was used to ground to fine powder the samples of flower buds but samples were ground to a fine powder in liquid nitrogen in a cooled pestle and mortar and allowed to stand briefly at room temperature while excess liquid nitrogen volatised. RNeasy Plant Mini Kit (Qiagen) with the addition of 45 µl (10% v/v) RNA Isolation Aid (Ambion Life Technologies Ltd, Paisley, UK) for more efficient removal of common contaminants of plant RNA preparations (e.g., polysaccharides and polyphenolics) and 4.5 µl (1% v/v) β-mercaptoethanol (Sigma-Aldrich Co. Ltd, Gillingham, UK) to 450 µl RNeasy® Lysis Buffer (RLT).

Crumbly fruit induction experiments

One hundred long canes, cultivar Glen Ample, were purchased from the same propagator (EU plants Ltd, UK); the plants were delivered in pots, each containing two long canes. For the experiments in the growth room and glasshouse, plants were left in pots, whereas for the field experiments they were planted in the soil. The first twenty-six plants were delivered at

the beginning of January and were moved into the growth chamber for the first two weeks, to allow acclimatization, with the following conditions: temperature (min. 10°C and max 14°C), humidity (70% R.H.) and daylight (16 hours). After fourteen days, the conditions in the environmentally controlled chamber were modified and in particular: max temperature was increased from 14 to 19°C and the day length brought from 16 to 18 hours per day; the hypothesis here is that the plants under these conditions would develop fruit with the normal phenotype. Under these conditions the plants started to blossom quickly. Some of the flowers were harvested for the hand pollination of the emasculated flowers and since pollen could have been a limiting factor of the experiments, all the flower buds from the top shoots were only used as a source of pollen. Once picked up, the flowers were dried at room temperature for two days and then stored in the fridge inside Petri dishes. For the growth room experimental campaign two set of pollen, Glen Ample and mix (Glen Moy, Glen Dee and Latham), were tested; here the hypothesis is that **xenogamy** (fertilisation of a flower by pollen from a flower on a genetically different plant) gives rise to berries with higher number of drupelets compared to **autogamy** (self-pollination of a flower) and/or **geitonogamy** (the fertilisation of a flower by pollen from another flower on the same or a genetically identical plant). The twenty-six plants were split randomly in two groups and half of them where pollinated with Glen Ample pollen and the other half with mix pollen.

Once the plants were established under these conditions, all unopened flowers one day from *anthesis* were emasculated; the emasculation were performed according to the method available at James Hutton Institute (N. Jennings, JH Ltd. Raspberry breeder *pers. comm.*). Flower buds were emasculated by means of a scalpel by simply following with the blade the contour of the five sepals, in this manner: petals, sepal and stamens were cut away. After two days, to allow the stigmas to get receptive to pollen, the emasculated flowers were first damaged and then hand pollinated. Six different treatments have been designed:

1. patchy (**P**)
2. core & tip (**C+T**)
3. base & tip (**B+T**)
4. base & core (**B+C**)
5. over pollination (**OP**)
6. receptacle (**R**)

The first four treatments are for the physical removal of stigma and style in four different specific patterns:

1) For **P** random removal of styles,
2) For the **C+T** treatments the styles were taken out from the top and the core of the receptacle leaving only those at its base,
3) For the **B+T** the styles were removed from the base and top leaving on the receptacle only those at its core while,
4) For the **B+C** the styles were cut off at the base and core of the receptacle leaving on it just those on the top.

These operations were carried out by means of a tweezer. The damage of the receptacle was accomplished by pinning its tip and side with a needle while the over pollination treatment, was designed to simulate the over visiting of the flower by pollinators and in this case the brush was simply rubbed nine times, both clockwise and anticlockwise, over the emasculated flower.

For each series of treatments a control was carried out by simply hand pollinating with a brush the emasculated flowers. Each treated bud was tagged with a strung ticket reporting the date of the emasculation, the kind of treatment carried out on it and the number of the plant; this information helped during the storing of the samples in the -80°C freezer. The same procedure has been followed for the other two experiments in the glasshouse and the field. These experiments are still on-going so no results are available at this stage.

The strong winds blowing over the field prevented the use of the strung tickets as they were all tore apart the following day; for those trials a plastic lace bent over a later shoot, was used to mark the samples.

Hormones extraction

Frozen samples from -80°C of: leaves, drupelets and receptacle for the last two samples were collected at three different stages: green, white and red corresponding to the stage of ripening of the berries being green, the immature, and red, ripened stage, respectively while white as the intermediate stage. Samples (4 replicates) were fine grounded in liquid nitrogen using a mortar and pestle, 100 mg of this fine powder was weighted in a 1.5 mL tube and mixed with 750 µL of cold extraction buffer (methanol:water:acetic acid, 80:19:1, v/v/v). Samples were shaken and extracted at dark for 16 hours and after that centrifuged at 16,000 g and 4°C for 5 minutes. The supernatant was transferred into a new 1.5 micro-centrifuge tube and dried in speed vac. After drying, 100 µL of MeOH were added to each sample, homogenized under vortex and centrifuged at 16,000 d and 4°C for 10 minutes. The supernatant was analysed by HPLC-MS/MS.

Reversed-phase high-performance liquid chromatography

Plant hormones were separated by an HPLC equipped with a reverse-phase column (C18 Gemini 5µm, 150 x 2.00 mm, Phenomenex, CA, USA) using a binary solvent system composed of water with 0.1% HCO_2H (**A**) and MeOH with 0.1% HCO_2H (**B**) as a mobile phase at a flow rate of 0.3 mL/min. Separations were performed using a gradient of increasing MeOH content. The initial gradient of methanol was kept at 30% for 2 min and increased linearly to 100% at 20 min.

Results

Gene expression: crumbly fruit potential genes to be validated via qRT-PCR

Microarray data that had not previously been studied were available from crumbly and non-crumbly fruits of a population of 188 individuals from a full sib family of 330 progeny derived from a cross between the European red raspberry cv. Glen Moy and the North America red raspberry cv. Latham. The data was analysed with MapMan software (Thimm O. et al., 2004). This program displays large data sets onto pictorial diagrams called bins that symbolically depict areas of biological function (i.e. a sector of metabolism, a cellular function, a biological response). Using this tool, eight genes were selected as follows; the main criteria for their choice have been the assigned function of the genes and their level of regulation comparing to the microarray data of crumbly and non-crumbly fruit.

1. gene28952-v1.0-hybrid encodes a Protein Phosphatase 1 regulatory subunit 7 (probable)
2. gene30607-v1.0-hybrid encodes a gibberellin 2-beta dioxygenase (putative)
3. gene22519-v1.0-hybrid encodes a Lipoxygenase A (putative)
4. gene22465-v1.0-hybrid encodes an endochitinase 1 precursor (putative)
5. gene09222-v1.0-hybrid encodes a transcription factor bhlh091 (probable)
6. gene18500-v1.0-hybrid encodes a nuclear transcription factor Y subunit C-2
7. Gene04423-v1.0-hybrid encodes a transcription factor MYB39
8. gene10913-v1.0-hybrid encodes a NIEMANN-PICK C1 protein precursor (probable).

The first four genes are involved in the hormones metabolism so they could be easily related to this condition since it is known that hormones are responsible for fruit set and development. The other three are transcriptional factors, quite generic but all are highly up-regulated in the

crumbly fruits compared to the normal fruit phenotypes, The last gene, with unknown function, is 120 times down-regulated compared to non-crumbly fruit.
The level of expression of these eight genes will be validated by running qRT-PCR.

RNA extraction from

Forty-five samples, three different stages: open flower bud (**OFB**), green berry (**GB**) and red berry (**RB**) from crumbly fruits plants grown in the field were available in the -80°C. RNA was extracted by using the procedures described in the material and methods of this report. The results are reported in the table below.

TABLE 1: Amount of RNA ng/µL extracted from the 45 samples

RNA EXTRACTIONS					
OFB [ng / µL]		**GB [ng/µL]**		**RB [ng/µL]**	
OFB 13	559.1	G 13	9789.1	R13	2628.7
OFB 50	928.1	G50	12201.2	R50	236.2
OFB59	1138.4	G59	9545	R59	1907.7
OFB 62	847.6	G62	303.3	R62	691.4
OFB 94	571.9	G94	1158.4	R94	124.1
OFB 103	1094.9	G103	3268.1	R103	580.4
OFB 118	326.3	G118	1245.2	R118	2402.5
OFB 126	548.4	G126	2215.6	R126	757.1
OFB 168	1961.1	G168	3658.4	R168	414.9
OFB 171	905.6	G171	1320.3	R171	1597.8
OFB 173	635.2	G173	1853	R173	970.8
OFB 181	766.3	G181	9693.6	R181	1370.6
OFB 195	836.7	G195	694.9	R195	2362.6
OFB 196	671.9	G196	3249.2	R196	2746.2
OFB 260	1215.3	G260	2242.2	R260	346.5

Crumbly fruit experiments

So far complete data are available only for the experiments carried out during winter inside the growth chamber. In total 520 flowers have been emasculated, the main target was to obtain at least four replicates of each treatment per plant; this would have allowed 2 samples of each treatments for both the two fruit stages, green and red berries, at which the samples were collected. Unfortunately not all the twenty-six plants gave enough flowers to be treated, oddly those placed in the centre of the first row, four plants in total, did not produce flower buds at all; the remaining plants gave enough flowers to get more than one replicates for both the two fruit stages. The samples have been collected daily and stored at -80°C and they will be used for the hormones profiling analysis this autumn.

Analysis comparing methods for producing crumbly fruit (growth room campaign)

Each response to the designed treatments (crumbly, normal, no fruit) was analysed relative to the number of treated buds using a generalised linear model with a binomial distribution and logit link function. The dispersion was estimated from the data.

For each response there was a significant effect of treatment, but not of pollen type. There was no significant interaction between treatment and pollen type. Because of this, and because there are many 'all or none' responses, predictions were made after fitting a model with treatment only.

TABLE 2: Predicted proportions crumbly fruit induction experiments growth room

Treatment	Crumbly	s.e.	Normal	s.e.	No fruit	s.e.
Base &Core (**B+C**)	0.9868	0.01106	0.0000	0.000055	0.01316	0.01017
Base&Tip (**B+T**)	0.9872	0.01078	0.0000	0.000053	0.01282	0.00992
Core&Tip (**C+T**)	0.9865	0.01137	0.0000	0.000055	0.01351	0.01046
Control (**C**)	0.0986	0.03000	0.8446	0.02525	0.05634	0.02139
Over Pollination (**OP**)	0.1034	0.03391	0.8599	0.026752	0.06897	0.02601
Patchy (**P**)	0.8947	0.02985	0.0239	0.009741	0.07895	0.02418
Receptacle (**R**)	0.9651	0.01678	0.0000	0.000053	0.03488	0.01546

s.e. Standard error

Focusing on the crumbly fruit, treatment was significant with $p < 0.001$.

The treatments fell into 3 groups:

I. Control and Open Pollinated **(OP)**. with least crumbly fruit

II. Patchy (**P**), with significantly more crumbly fruit than control and Over Pollination (**OP**), but significantly less than the other treatments.

III. Receptacle (**R**), Core and Tip (**C+T**), Base and Core (**B+C**), Base and Tip (**B+T**), with most crumbly fruit. There are no significant differences within this group.

The other note-worthy comparison is with regard to "No fruit": this is highest for Patchy (**P**), and significantly greater than for Base and Tip (**B+T**), Base and Core (**B+C**) and Core and Tip (**C+T**).

Crumbly fruit new definition

Another important result in this first year of the Ph.D. is the development of a new and more standard definition of the crumbly fruit which considers the different level at which the condition reveal itself. The Crumbly Fruit Condition (**CFC**) is linked with pollen abortion and embryo sac degeneration causing drupelets to be generally reduced in number but greatly enlarged or, in case of small reductions, cohere imperfectly so the fruit crumbles when is picked up (Graham et al., 2015). Only plants where all the fruits are symptomatic year after year are affected by this undefined disease or genetic disorder.

Plants that display malformed fruit which look similar to crumbly fruit in their appearance but where the symptoms are intermittent within a year or over the subsequent years are now defined as plants with symptoms of Malformed Fruit Disorder (**MFD**).

Malformed Fruit Disorder is present when plants display uneven fruit set, generally at the very beginning of fruit production and this occurs mainly on the top lateral shoots. Fewer symptoms are observed as the season progresses and more flowers and fruits are produced by the plant resulting in little or no loss of yield.

This condition can be more severe when malformed fruits are observed throughout the entire fruiting seasons and displayed on most or all the laterals however these plants do not display the symptoms every year.

Discussion

Crumbly fruit induction

In Year one the aim was to be able to determine if the crumbly phenotype can be triggered, plants were treated in a number of ways ranging from treatments with no damage to those causing severe damage to compromise the normal development of the fruit in an attempt to induce the crumbly fruit. As stated in the introduction raspberry fruit is quite complex being an aggregation of multiple fertilized ovaries; to grow properly at least ¾ of the carpels must be fertilized by pollen and as we cannot anticipate that they will all get pollinated at the same time, there must be some mechanism that controls the simultaneous development of the fertilized ovaries. An important role in the crumbly fruit may be played by the receptacle which could trigger the physiological processes leading to fruit setting. No data are available in the scientific literature, thus we cannot omit the role of the receptacle in the process of fruit setting and development. A second hypothesis in this research was that there is a system, a crosstalk between fertilized ovaries and the receptacle that synchronize the development of the fertilized ovules. According to this hypothesis, once fertilized each ovary sends a molecular message (i.e. a hormone signal) to the receptacle that acts as a lead hub, where it

accumulates such cues and as soon as it receives a minimum number of molecular signals, corresponding to at least to ¾ of the flower carpels being fertilized (under this number a proper berry cannot develop) the receptacle then sends a simultaneous molecular message to all the fertilized ovaries that triggers their growth. On the basis of this assumption six different treatments were proposed to interfere with this growth regulating system causing the fruit to get crumbly.

The first four treatments involve the physical removal of stigma and style following a specific pattern for each of the treatments but in all the cases less than ¾ of the carpels was kept intact. This was to interfere upstream with the hypothetical fruit set process. On the other hand, the damaging of the receptacle was going to compromise the process downstream since once damaged the receptacle was not be able to lead the regulating process. Over pollination was designed to simulate the pollination process carried out by insect pollinators. Its aim was to study and better understand the role played by these animals in the crumbly fruit disorder. This was achieved by repeated hand pollination (i.e. 9 times more compared to the two hand pollinations of the other treatments) to mimic the natural phenomenon which occurs when pollinators, bees mainly, visit the same flower more than 4 times, resulting in damage to flowers. For each series of treatments a control was carried out, using the standard protocol for hand pollination already performed at the Institute in the Raspberry Breeding Programme, namely emasculating the flower bud when it is still closed and then, after two days, hand pollinating the carpels once a day per two days in a row. Such experiments should be of help:

a) Understanding if damage is a factor in crumbly fruit;

b) If there is a crosstalk between the fertilized ovaries at the beginning of fruit setting, as it is unlikely that all the ovaries get pollinated and then fertilized at the same time, so a sort of signalling between the ovaries must be considered;

c) To better comprehend bee pollination efficiency and how to eventually control it.

The results of the first experiments to induce crumbly fruit, those performed in the growth room, show that all the designed treatments, except the over pollination OP, can cause crumbly fruit. These results prove that phenomena interfering or compromising ovule fertilisation, the removal of styles and stigmas (**P**, **B+C**, **B+T** and **C+T** designed treatments) are within those, can be regarded as potential triggers of **CFC** (Crumbly Fruit Condition) and/or **MFD** (Malformed Fruit Disorder). This was already observed in plants infected by Raspberry Bush Dwarf Virus (**RBDV**; Isogai et al., 2014); or, as recent field observation suggest, could be assumed for the nectar whose dripping and accumulation over the stigmas made them impossible to receive the pollen and then affecting the fertilisation of the ovules.

Alternatively, the damage of the receptacle (**R** treatment) can trigger **CFC** and/or **MFD** since according the proposed hypothesis, the receptacle acts as a leading centre (hub) to synchronize and regulate the growth of the fertilised ovaries then once wound it cannot carry out its function causing the failure of that supposed molecular coordinating system and the subsequent unsynchronised growth of the fertilised ovaries resulting in fruits to be misshapen.

Hormones Profiling – Development of a Methodology:

As described in the introduction, many hypotheses can be proposed for the triggers of the crumbly condition in raspberry; some of the main factors that may be responsible for causing the crumbly condition in raspberry are failed pollination, damaged flowers, adverse environmental conditions and/or plant pathogens.

Each of these stresses (abiotic and biotic), alone or in combination, are responsible for the activation of signalling pathways which involve intermediates and crosstalk through: hormonal, redox and enzymatic components. Understanding the hormones involved in fruit set together with those activated during stress conditions, in crumbly and non-crumbly fruit, is a key factor to better understand the trigger(s) of such condition and possible find solutions to monitor and then avoid this disorder. In year one another aim of the project is to develop a protocol suitable for LC-MS which will allow hormone profiling to be performed on: flower bud, receptacle and drupelets (these last two at different growth stages i.e. green berry and red berry).

To develop the hormones profiling method, two papers (Pan X. *et al*., 2008; Trapp M.A. *et al*., 2014) were examined. The first being a valid and robust method for the chromatographic separation of the 17 different hormonal compounds : salicylic acid (SA), benzoic acid (BA), indole-3-acetic acid (IAA), indole-3-butanoic acid (IBA), indole-3-carboxylic acid (ICA), abscisic acid (ABA), cinnamic acid (CA), gibberellins A_3 (GA_3), gibberellins A_4 (GA_4), jasmonic acid (JA), 13-epi-12-oxo-phytodienoic acid (OPDA) were analysed in negative scan mode as $[M-H]^-$ while others (salicylic acid methyl ester (MeSA), indole-3-acetic acid methyl ester (MeIAA), cinnamic acid methyl ester (MeCA), jasmonic acid methyl ester (MeJA), benzoic acid methyl ester (MeBA) and zeatin) were analysed in positive mode as $[M+H]^+$. The other scientific paper (Trapp M.A. *et al.,* 2014) is more reliable and optimized for what concerns the phytohormones extraction and the validation of the compounds molecular mass determination.

Both the two reviewed methods have been developed to analyse the extract from leaves while this work is concerned with flower and fruit. The first step is to develop an extraction method suitable for these samples (i.e. receptacle, drupelets and bud). Pan X. et al., for the sample preparation used 50-100 mg of fresh material sealed in 1.5 mL snap-cap vials. After being

frozen in liquid nitrogen the leaves were grounded and suspended in 500 µL of 1-propanol/H_2O/concentrated HCl (2:1:0.002, vol/vol/vol) followed by agitation for 30 minutes at 4°C. After this 1 mL of CH_2Cl_2 was added followed by 30 minutes of agitation and then 5 minutes of centrifugation at 16,000 g. The centrifugation forms two phases with the plant debris in the middle of the two layers. The bottom layer is the one of interest and must be concentrated and re-solubilized in MeOH; 25 µL to be injected to column analysis. This extraction protocol was followed for the fruit samples with poor detection for some of the compounds. A sample of the top layer of plant extract was found to contain those phytohormones, poorly detected in the lower solution. Trapp A. *et al.,* (2014) evaluated two different parameters during the optimization of their phytohormones extraction method: composition of the extraction solution and type of plant sample (fresh or dry material). Tubes containing 100 mg of plant material were kept at -80°C or dried overnight in a freezer drier at -42°C. The extraction wass performed adding 1 mL of either: ethyl acetate, dichloromethane, isopropanol, MeOH or MeOH:H_2O (8:2) into each tube containing dry or fresh plant material. Samples were shaken for 30 minutes and then centrifuged at 16,000g and 4°C for 5 minutes. The supernatant was transferred into a new 1,5 micro-centrifuge tube and dried in speed vacuum. After drying, 100 µL of MeOH was added to each sample, homogenized under vortex and centrifuged at 16,000g and 4°C for 10 minutes; the supernatant was then analysed by HPLC-MS/MS. The authors evaluated the influence of both MeOH:H_2O ratio and the addition of acid in the extraction mixture using three different MeOH:H_2O ratios (7:3, 6:4 and 1:1) pure, or containing 0.2% of HCl. The mixture of MeOH and water provided higher extraction efficiency for all phytohormones and in particular the ratio of 7:3 has been chosen as extraction solution due to its good performance in extracting, on average, all the selected phytohormones. Trapp A. *et al.,* (2014) method concerns the optimization of the mass spectroscopy parameters. This technique will be used in this research to identify the phytohormones in fruit samples; the only limit of this method, is that the protocol has been developed for the analysis of only 6 phytohormones and not 17 as done in the method of Pan X. *et al.,* (2008). Thus a new protocol has been established for the hormones profiling based on Pan X. *et al.,* (2014) for the liquid chromatography elution of the plant extract while Trapp A. *et al.,* (2014) method will be used for samples extraction and mass spectrometry analysis. Initially samples will be analysed from berries both crumbly and non-crumbly already available at JHI from previous studies. Once established the new protocol will be used to analyse samples from the Glen Ample long cane damage experiment aimed at inducing the crumbly fruit condition. The protocol allows the simultaneous determination of seventeen different hormones (Zeatin, Indole-3-carboxylic acid, Indole-3-acetic acid, Indole-3-butyric acid, Indole-3-acetic acid methyl ester, Jasmonic acid, Jasmonic acid methyl ester, Salicylic acid, Salicylic acid methyl ester, Benzoic acid, Benzoic acid methyl ester, Cinnamic acid, 12-oxo-

epi-Phytodienoic acid, Gibberellin A3, Gibberellin A4 and Abscisic acid). All seventeen standards that can be detected with this LC-MS method have been purchased and two cocktail solutions prepared, one for the negative ionization mode containing: GA_3, ICA, IAA, BA, ABA, SA, CA, IBA, JA, GA_4 and OPDA; while the second for the positive ionization mode containing: zeatin, Me-IAA, Me-BA, Me-SA, Me-CA and Me-JA; both the solutions were prepared with a hormone concentration of 100 µM.

Understanding of the hormones potentially involved in crumbly fruit will be utilised to set up a second phase of specific experiments of plants cultivated in the growing chamber where experiments will be carried out to induce the crumbly condition chemically via hormones spraying, individually or in combination depending on the results from the initial hormone profiles on crumbly and non-crumbly fruit. As there is a systematic and consistent increase in drupelets number when plants are developed in short day (SD) and low temperature (LT) conditions during flower induction and female sex expression is generally favoured by SD and LT, these environmental effects are responsible, at molecular level, for changes in the endogenous plant hormones levels with auxin favouring female and gibberellins male sex expression. The crumbly condition could therefore be the result of plants having disproportionate rate pistils/stamens. It is possible that a very low number of pistils per flower (decreased femaleness), paired or not with a high number of stamens, could be the simplest scenario responsible for the crumbly condition. Thus as long as not enough ovaries get fertilized, the fruit can be misshapen. Since the exact physiological processes leading to fruit set is not known, it is not possible to omit other scenarios such as decreased masculinity and or partial parthenogenesis phenomena in the fruit set process.

This last scenario seems to be the less probable since in a preliminary experiment involving 18 number of fruits of cv. Adelita, for this work the number of drupelets and then the number of seeds from the same fruit was determined. Here seeds were extracted by squeezing the drupelets, previously separated one by one and counted, in a small colander under a very low water flow; once all the traces of the fleshy part of the fruit were removed, the seeds were dried on a tissue and then counted. No differences were found, indicating that parthenocarpic process was not occurring during fruit setting in raspberry. Eventually a larger study considering a much larger population could be carried out to get a significant statistical evidence for the lack of parthenocarpy.

Gene Expression Experiments

RNA from 15 samples collected from the population of plants from the cross between Glen Moy x Latham and differing in crumbly phenotype has been extracted. The samples include three different development stages: open flower buds, green fruit and red fruit.

For the first stage, flower buds, the extraction process was performed with Qiagen RNeasy® plant mini kit. For the other two stages the Qiagen kit was less successful as these samples were rich in polysaccharides and polyphenols both classes of molecules interfere with the RNA extraction and therefore a protocol involving phenol:chloroform:IAA was used which resulted in enough RNA to be extracted; The protocol developed here was based on Zumbo (2010).

This method uses a Phenol:Chloroform mix, phenol being efficient at denaturing proteins. The phenol-chloroform combination reduces the partitioning of poly(A)+mRNA into the organic phase and reduces the formation of insoluble RNA-protein complexes at the interphase. Phenol retains about 10-15% of the aqueous phase, which results in a similar loss of RNA; chloroform prevents this retention of water and thus improves RNA yield. Purified phenol has a density of 1.07 g/cm^3 and therefore forms the lower phase when mixed with water (1 g/cm^3). Chloroform ensures phase separation of the two liquids because it is miscible with phenol and it has a higher density (1.47 g/cm^3) than phenol; for such reason, chloroform forces a sharper separation of the organic and aqueous phases assisting in the removal of the aqueous phase with minimal cross contamination from the organic phase. In general, solutes dissolve better in a solvent that is more similar in chemical structure to itself, a very polar solute is more soluble in high polar water than in fairly polar methanol and almost insoluble in non-polar solvent such as chloroform. Nucleic acids are polar because of their negative charged phosphate backbone then they are soluble in the upper aqueous phase instead of the lower organic phase (water is more polar than phenol). Conversely, proteins contain varying proportions of charged and uncharged domains, producing hydrophobic and hydrophilic regions, in presence of phenol the hydrophobic cores interact with the solvent causing the proteins and polymers, including carbohydrates, to precipitate and to be collected at the interphase between the two phases or for the lipids to be dissolved in the lower organic phase. Moreover the pH of the phenol determines the partitioning of DNA and RNA between the organic phase and the aqueous phase. At neutral and/or slightly alkaline conditions (pH 7-8), the phosphate diesters in the nucleic acids are negatively charged, and thus DNA and RNA both partition in the aqueous phase; DNA is removed from the aqueous layer as the pH is lowered with a maximum of efficiency at pH 4.8. On the basis of these considerations a protocol for the extraction of RNA from red and green berries which allowed good quality RNA from samples of red and green berries to be extracted. The integrity of each RNA has been determined by 2% (w/v) agarose gel electrophoresis.

In order to validate the expression levels of these eight selected genes from microarray data, primers and probes have been designed for all the eight candidate genes using the Universal ProbeLibrary Assay design software (Roche) to ensure common thermal cycling parameters, for the transcript normalization in *Rubus*, three reference genes: **TIP41-like family protein**,

YLS8 thioredoxin-like U5 snRNP and **Clathrin adaptor subunit** have been chosen since they were already tested in previous gene expression experiments on raspberry fruits (Simpson *et al*., 2016).

Crumbly fruit new definition and questionnaire for growers

The evidence of crumbly fruit developing over time, especially in the last 5 years, created new challenges for The James Hutton Institute, in particular for what concerns the release of material for the propagation industry; JHI is the sole source of *Rubus* plant material for entry into the UK Plant Health Certification Scheme. For some varieties in the last few years the situation worsened and in some cases, the release of health material for the propagators became really challenging; the lack of a reliable test method to screen crumbly fruit did not help. For these reasons the JHI decided to start a partnership with Naktuinbouw, its Dutch equivalent to see if together they could find a solution to this issue. In Holland they work with raspberry and they are facing the same problem with crumbly fruit as in Scotland. As part of this collaboration, there was a visit last May to their laboratories and facilities. During the guided tour through their glasshouses, dedicated to the crumbly fruiting testing; it became apparent that a different way was needed to classify the condition. The first step was to develop a standard definition for the crumbly fruit since in order to solve a problem it must be clearly defined. What was beyond any doubt to both sides was that crumbly fruit is principally a description of symptoms rather that just an undefined disease or a genetic disorder and it can arise at different levels. For this reasons the standard concepts of **MFD** (Malformed Fruit Disorder) and **CFC** (Crumbly Fruit Condition) were introduced both described in the previous chapter.

Another important aspect to consider is to get a complete overview about the crumbly fruit phenomenonof its distribution worldwide. It would be very interesting and useful to know the varieties that are more prone to **MFD** and/or **CFC** and where, the geographical area, are cultivated. This could then be linked to weather and soil type. This would allow an estimate of how the genetics of raspberry and the environment influence crumbly fruit development.

The first idea to get these data was to contact via email public bodies (i.e. minister of agriculture) of each single state, obviously concentrating on countries with a florid raspberry industry. Unfortunately, from a first check, by browsing in the goverment official web sites of Italy, UK and USA, it was clear that no specific data are available; there is no official monitoring of crumbly fruit at all. A further attempt could have been done by contacting the industries of each single country to see if they have data but this would have requested a large network of contacts and then the chances that they will be willing to share their information.

Last July during the first meeting with the Ph.D. industry supervisor, the idea to create a specific and well informative database about crumbly fruit, by making an electronic survey (for example using surveymonkey) for the raspberry growers to be completed, arisen. In fact developing an easy and well organized questionnaire with focused questions would allow to get information not limeted to: variety grown, weather and and eventually soil characteristic but extended to very detailed aspect of the whole cultivation process such as: agronomic practises, condition of the plantation (i.e. age of the plants), origin of the plants (i.e. root or micro propagated), certification statutus of the plant (i.e. plant health certificate) and so on. Obviously the questionnarie would go with a brief introduction of myself and of the Ph.D. research project; it is mandatory to convince growers that the survey is important to them too and it is not just a chapter to fill my thesis. Together with this introduction, the new definition of **CFC** (crumbly fruit condition) and **MFD** (malformed fruit disorder) would be provided and in the questionnaire, as first enquire would be asked if they have ever found in their plantations, plants showing **CFC** and/or **MFD**. The idea is to keep the information collected anonymised since the common sense says that people, and in this case growers, are likely to be much more open if they know their data is confidential.
A first draft of the questionnaire is ready and will be reviewed in October and the survey will be launched in the second half of November when the raspberry growing season will be over, at least in the boreal hemisphere, and farmer will be likely to respond. Questionnaire and new definitions will be translated in as many possible languages in order to spread it over countries where English is not known by the majority of growers (i.e. South of Europe, China, Nord Africa).

Conclusions

The first experiments to induce crumbly fruit condition (growth chamber) proved to be successful (see chapter discussion for more details), the first results from the other two experiments, glasshouse and field, seem to be very encouraging too. It looks like that is possible to induce crumbly fruit by mechanical damaging the flower or more specifically by reducing the number of carpels available for pollination and or by spoiling with a needle the receptacle. The first assumption that can be made is that all phenomena interfering or compromising ovule fertilisation and its subsequent development can be regarded as potential triggers of crumbly fruit.
Raspberry Bush Dwarf Virus (RBDV) negatively affects pollen tube elongation, it does not grow enough to reach the ovary, and indirectly prevents the fertilisation of the ovule. Obviously if less ovules get fertilized consequently a lower number of drupelets develop and then the risk of berries misshapen and/or crumbly is higher.

During the observation of the plants both in field and glasshouse, we have noticed that another interesting factor that seems to be related with crumbly fruit condition is the flower nectar; if not collected by pollinators it drips quite abundantly over part of the carpels, the stigmas get impregnated by this sticky substance and cannot be reached by pollen. Again another situation where potentially a reduced number of ovaries get fertilized resulting in berries with reduced number of drupelets or in the worse scenarios becoming misshapen or crumbly.

RBDV infections, flower mechanical damaging (carpels or receptacle) and flower nectar, even though acting in different way, can all be responsible of misshapen fruit syndrome and or crumbly fruit condition. There is extensive evidence for the role played by viruses in the literature. The effect of mechanical damaging has been proven in these experiments. The influence of flower nectar, so far, can be only hypothesized since it is just a result of my examination and for this reason a new experiment has been designed and will be carried out next spring where two different pollinators, honey bees and bumble bees alone and in combination, will be tested to work out the best system to avoid flower nectar dripping.

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