Leaf edge senescence on watercress crops in southern Spain; cause and potential solutions.

Industry Report.

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Abstract

Watercress grown in Spain can be adversely affected during the winter from driving rain, hail or other weather events including low temperature (especially when frosts are experienced over a number of days). Leaf edge senescence is observed on crops during mild and humid periods and is exacerbated following abiotic stress. Here we look at causes of leaf edge senescence and evaluate potential control methods.

Influencing factors may be both physiological and pathogenic. As such, factors including humidity and leaf structure are looked at as is nutritional status of healthy leaves and leaves showing symptoms. Crops affected are screened for pathogens and pathogenicity. Bacterial pathogens are investigated since symptoms seen on crops in Spain are similar to those cited as being caused by bacterial infections in watercress crops. Symptoms cited are those starting on the leaf edge as seen on watercress crops in Hawaii, McHugh and Constantinides (2004). Roberts (1999) describes symptoms of bacterial infection as wedge shaped necrotic patches originating from the leaf margin. Potential control methods are reviewed.

Xanthomonas nasturtii, not previously recorded in Europe was isolated from crops in Spain showing symptoms of leaf edge breakdown at 3 separate institutions; Fera (York), Cambrico Biotech (Seville) and The University of Warwick. Pure isolates from Warwick were used to test for pathogenicity on watercress plants in Spain and at Warwick University. *Xanthomonas nasturtii* was also identified on seed (seed produced in Spain) and *Xanthomonas spp*. in water recirculating through the beds. Leaf edge breakdown was found to start at the leaf edge hydathodes and initial damage appears to be exasperated by humid conditions. Following identification, potential control options for *Xanthomonas nasturtii* were looked at both *in vitro* as well as in field trials. Control using the sterilant hydrogen peroxide on seed was assessed as was Ultraviolet (UV) water treatment of recirculating water.

Xanthomonas nasturtii showed symptoms of pathogenicity following inoculations on watercress plants; a higher level of pathogenicity noted on crops untreated against those treated with copper gluconate. Copper gluconate inhibited bacterial growth *in vitro* to varying degrees. Crops treated during field trials showed reduced disease incidence at times of year when crops have experienced abiotic stress, humidity is high and/ or rainfall noted and temperatures are mild. Hydrogen peroxide reduced levels of E. coli and pseudomonas on seed. UV affected E. coli levels in recirculating water but had no effect on *Xanthomonas spp.* It would be useful to look specifically at *Xanthomonas* levels pre and post sterilant application on seed and investigate further why UV had no effect on *Xanthomonas spp*. in water.

Key words; Leaf edge senescence, watercress, *Xanthomonas nasturtii*, copper gluconate

Word count: 7230.

Introduction

Watercress (Nasturtium officinale), a fast growing perennial dicotyledon naturally found growing on the edge of streams, is one of the oldest plants utilised by man. It belongs to the same family (brassicaceae) as other important food crops such as cauliflower, sprouts and cabbage. Watercress has a very specific growing condition; in fact it is classed as an aquatic vegetable by the EU due to it being grown in flowing water.

This crop has been grown commercially in the UK since the 1800´s (concentrated in the south of England in the Dorset chalk belts as well as Wiltshire and Hampshire) and in southern Spain since the mid 1980´s. It was at this time that, with pressure for all year round supply to the UK supermarkets, The Watercress Company based in the UK expanded its production area to the south of Spain. Crops are grown here to cover supply during the winter months as well as provide back up during the early summer months.

Watercress is grown in Spain on gravel beds (figure 1); its roots taking nutrients directly from the flowing recirculated water flowing through the beds on a slope of 0.5%. Production starts early September when beds are leveled, a small amount of water added and seed drilled. Crops are then harvested up to 4-5 times through the winter until the initiation of flowering in the spring necessitates that these crops be replaced and re drilled. Winter production is shared between the Spanish (Jerez de la Frontera) and American farms (situated outside Tampa in Florida). Nutrients are both broadcasted over the beds and added in lines to the water recirculating through the beds.


(a)

(b)

(c)

(d)

Figure 1 Watercress crops in southern Spain (a) emerging from its gravel base 4-5 days post drilling; (b) Recirculating water entering the bed; (c) mature watercress crop; (d) Watercress crop being harvested.

During the period from November through to February, quality of crop can be affected through leaf senescence. This senescence usually can be seen first on the leaf margin, (figure 2) and, under favourable conditions, spreads into the leaf. Favourable conditions can include cloudy, humid periods through the months when days are shortest. Quite often senescence is seen when this mild, humid weather follows a period when minimum temperatures are low enough for frosts causing damage to the leaf waxy cuticle or the crop has experienced abiotic stress from other sources.

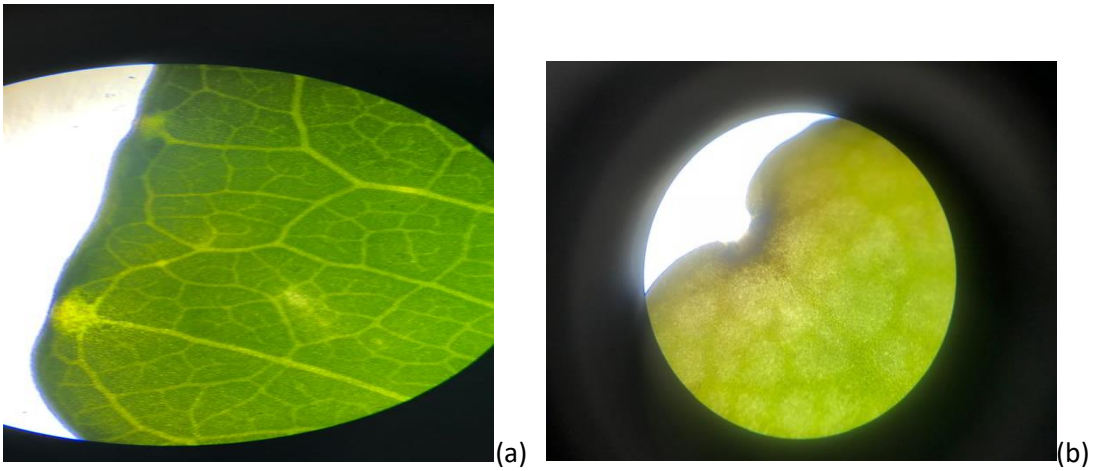

(a) (b)

Figure 2. Watercress leaf edges taken under microscope (a) Leaf edge hydathodes (b) Start of leaf edge senescence.

Leaf edge senescence can reduce yield by 40- 60 %. In severe cases can result in crop being rejected either at harvest or by the customer due to a reduction in post-harvest shelf life.

Senescence is first noted at the tips of leaves. Figure 2 shows (a) healthy leaf as seen under the microscope (b) early symptoms of leaf edge senescence. Howard and Lyon (1952) make reference to hydathodes being evident at these points and figure 2 suggests this is the case with watercress. Hydathodes are seen in many brassicacea plants including cabbage and Arabadopsis, Laureano et al (2014). They are designed to remove liquids from the leaf during periods (such as night) when transpiration rates reduce but root pressure continues to enable the flow of fluids to the leaf. Cerutti (2017) describes the hydathode as a principle entry point for bacterial diseases. Influences on leaf deterioration and symptom development on watercress crops could be both physiological and pathological and, as such, solutions could involve both avoiding physiological damage to crops as well as disease mitigation and control. Understanding of the cause and evolution of symptoms would greatly assist in finding solutions, improving quality of crop harvested and potential improvements in shelf life whilst reducing waste.

Watercress crops in southern Spain are susceptible to disease pressure from a number of sources. Fungal infections such as cercospora leaf spot, alternaria have been identified on crops in Spain as has Watercress white vein virus which was first identified in 2014 on watercress crops. These have all been noted following a period when abiotic stress is experienced by the crop.

Further afield, *Xanthomonas campestris* has been cited as affecting crops in Hawaii especially during periods of high humidity or rainfall. *Xanthomonas nasturtii* was first described in 2017

Vicente (2017) from crops growing in Florida as a new pathogenic disease specifically affecting watercress. Vicente (2017) describes *Xanthomonas nasturtii* as gram- negative straight rods with a single polar flagellum. Colonies appear yellow when plated on yeast dextrose calcium carbonate agar for 48 hrs at 28°C. Symptoms are noted as leaf edge senescence developing into triangle necrotic patches in the leaf similar to symptoms seen on crops in Spain (described by Vicente (2017) in Figure 3).

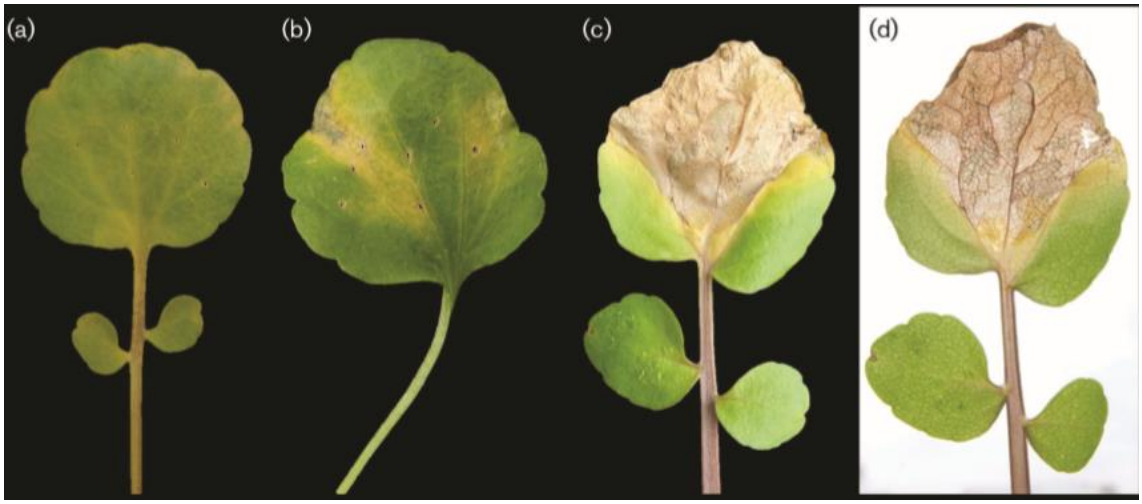


Figure 3 Watercress showing levels of Xanthomonas infection from a where the plant is free from infection to d where symptoms are severe, Vicente (2017).

Maji and Nath (2015) describe Xanthomonas campestris entering the plant via natural openings such as stomata or hydathode.

Leaf edge senescence in Spain can be observed during periods of high humidity and / or rain fall normally together with low air movement. McHugh and Constantinides (2004) refer to humid conditions favouring *Xanthomonas campestri* infection of watercress and spread assisted through splash from overhead irrigation. Bacterial infections are likely to be opportunistic and González- hernándes (2018) describes *Pseudomonas syringae* surviving on and in leaf in its epiphylic stage and when populations are high enough inside the leaf induces the expression of virulent factors and secondary metabolites that facilitate the colonization of the host and disease development occurs. Billing (1987) describes bacteria colonizing tissue around the hydathode where it has direct access to the xylem. Hugouvieux et al (1998) describes *Xanthomonas campestris* as being a vascular pathogen gaining entry into the plant via hydathodes. As other bacteria *Xanthomonas* is unable to actively enter the leaf so requires entry point such as hydathode, stoma or wound. Meenu (2013) describes infection of black rot, caused by the bacterium *Xanthomonas campestris* of cabbage though Hydathodes being seen late 1800´s. Meenu (2013) describes how *Xanthomonas campestris*, once inside the leaf surface exudes a polysaccharide xanthan which causes the xylem to become blocked resulting in the typical V- shaped chlorotic lesion described in figure 3 by Vincente (2017)

In order for bacteria to become pathogenic, they rely on elicitor proteins injected into plant cells being undetected by the plant in order to allow disease establishment (pathogenicity). If

these are recognised by the plant, this will result in inhibition of bacterial growth and thus resistance.

Plants show two types of resistance to disease; constitutive and induced. Constitutive resistance is that which involves responses to attack such as waxy leaf coating. Secondary metabolites can have an antimicrobial effect such as Flavonoids and Tanins (Phenolics). Defensive proteins can be utilised to break down components of pathogen cell walls. Induced resistance involves methods that are not normally switched on in the plant to save energy and are activated in response to attack such as cell wall thickening (lignification).

Copper oxychloride has been traditionally used to control bacterial infections in many crops. However, with its extensive use, resistant strains have been selected through repeat applications reducing the effectiveness of this active, Gonzalez (2018). Environmental concerns over increased levels of copper in soils where crops such as grapes and tomatoes have been grown over a period of time have also directed attention to look for alternative solutions. One such is copper gluconate, an organically approved active which is readily assimilated in the plant and shows no signs of phytotoxicity that copper oxychloride has, Gonzalez (2018).

Here we hypothesise that leaf edge senescence is brought about by pathogenic bacteria and that copper gluconate can limit the development of this disease on watercress crops in Spain.

Materials and methods

In order to attempt to induce leaf edge senescence, watercress plants were initially subjected to elevated humidity (up to 84% recorded by data logger) by covering crops growing in watercress beds with plastic bottles. Observations were made and where an effect was noted due to plants being covered, leaves were analysed by a lab in Seville (AGQ labs) for their nutrient status compared with unaffected leaves.

Samples of watercress plants showing leaf edge senescence were collected and screened for pathogenic bacteria. Initial samples were taken from crop in the field showing symptoms following a period of abiotic stress (low temperature) and sent to Fera, UK where partial gene (gyrB) sequencing was carried out. Further samples were sent to Warwick University from leaf marginal necrotic patches (observed after covering healthy crop with plastic for 4 days). These were plated and isolates obtained frozen and subsequently grown on at 28°c for 48 hours to obtain DNA samples. Molecular PCR (polymerase chain reaction) methodology was used using 16S ribosomal DNA sequences for identifying the genus and gyrB for identifying the species. This involved heating and cooling the DNA extractors with the polymers in order to rapidly multiply the quantity of DNA in the sample. The PCR products resulting were sequenced and compared with sequences available for identification. An isolate of bacteria obtained was sent back to Spain for further trial work.

Watercress seed as well as recirculating water from the production units in Spain were screened for bacteria by Fera in York, UK. Watercress seed was tested at Fera using methodology based on the ISTA validated system for Xanthomonas campestris identification.

This involves a minimum sample size of 30,000 seed and involves soaking the seed to extract the bacteria. Recirculation water was tested at Fera using Fatty Acid profile analysis of isolates exhibiting typical colony morphology, Fera (2019).

Pathogenicity tests were carried out on watercress plants in Spain as well as at Warwick University using the isolate obtained at Warwick University. In Spain, watercress plants were grown from seed which had been sterilised with hydrogen Peroxide before drilling. Plants were raised in commercial watercress beds and nutrition was supplied as per standard program for crops in Spain. 50% of plants were treated with a 6% concentration of copper gluconate (rate 1.8l/ha) at second true leaf stage (around 15 days from drilling) and again a week later at 5th true leaf stage. At this stage plants both treated and untreated were transplanted into containers containing potting compost and irrigated with distilled water. Crops were analysed for copper content 48 hours post treatment.

Xanthomonas bacteria solution was prepared with the assistance of Cambrico Biotech lab in Seville. Using a sterilised needle, two leaves per plant were wounded using the multi needle pricking method as described by Maji and Nath (2015). Inoculation involved dipping the needle in the bacteria solution prior to wounding (figure 4). A separate sterilised needle was used to wound control plants. Leaves wounded and inoculated were marked with coloured thread. 24 hours after inoculation, wounds where bacteria had been inoculated were dipped in bacterial solution as described by Maji and Nath (2015). Plants were covered with plastic covers to raise humidity levels. Average humidity over period of trial 87% (recorded using a data logger). Average temperatures during the trial were as follows; max 25°c, min 14°c.


(a)

(b)

Figure 4 (a) Inoculation of watercress plants (b) Inoculated leaves marked using coloured thread.

Here we compared plants (visual evaluation); treated and inoculated; treated and not inoculated (although wounded using a multi pin method but not applying the bacteria); not treated and inoculated; not treated and wounded. Observations after 10 days made comparing % of leaf affected by necrosis. Data was uploaded onto the statistical package R. Data was tested for normality and a box plot created to show treatment against level of necrosis. An analysis of variance (aov) was carried out to test for significance of treatment against level of necrosis.

Field trials were carried out using organically approved products sourced in Spain from the company Idai nature, Valencia. These included Idai Cobre (copper gluconate), Naturdai Mimetic Idai Brotaverd and Naturdai Citriseed.

Idai Brotaverd is a natural biostimulant reported to stimulate the production in the plant of its own phytoalexins and phenols (defence compounds). It is assimilated by the plant and, due to its vasodilator properties, reduces blockages caused by vascular diseases.

Naturdai Mimetic is based on extracts of Minosa tenuiflora (mimosa) plus Quercus robur (oak). This encourages a thickening of the cell walls. It is reported to both starve bacteria of its food source in the plant plus, with tannins present, triggers the plants own defence mechanisms which break down the bacteria membrane.

Naturdai citriseed is based on citrus seed oil. It is reported to trigger the production of exoelicitors in the plant which in turn help to form phytoalexins. The 3 weekly treatments were planned as in figure 5 below;

Treatment 1

week 1	Naturdai Mimetic 2 L/ha
week 2	Mimetic 2 L/ha + brotaverd 2 L/ha de caldo
week 3	Naturdai Mimetic 2 L/ha
week 4	Mimetic 2 L/ha + brotaverd 2L/ha de caldo

Treatment 2

week 1	Naturdai citriseed 2 L/ha
week 2	N. Citriseed 2 L/ha
week 3	Naturdai Citriseed 2 L/ha
week 4	N. Citriseed 2 L/ha

Treatment 3

week 1	Naturdai Mimetic 2 L/ha+ cobre 2l/ha
week 2	Mimetic 2 L/ha + cobre 2 L/ha de caldo
week 3	Naturdai Mimetic 2 L/ha+ idia cobre 2l/ha
week 4	Mimetic 2 L/ha + cobre 2L/ha de caldo

Figure 5; Treatments were made to whole watercress beds (1600 m2 each)

Evaluations made weekly as follows;

- Severity of leaf edge senescence of 10 randomly collected plants (selecting samples in a W pattern through the beds).

Weather data was recorded throughout the trial. Data obtained was uploaded onto the statistical package R and plotted on a box plot to show bed against % breakdown severity. The non-parametric Kruskal- Wallis test was carried out looking at the significance of date on senescence and a Friedman test looked at location against senescence.

Complimenting field trials, *in vitro* testing was carried out in order to look at a number of actives and their effectiveness in inhibiting the growth of the isolate obtained from Warwick originating from necrotic lesions on watercress in Spain. Actives included copper gluconate, bleach, hydrogen peroxide and bacillus subtilis. *In vitro* testing was carried out at Cambrico Biotech, a diagnostic laboratory in Seville, Spain. Kings Medium B agar was prepared by the

laboratory technicians spread over sterilised petri dishes and left to set. Bacteria isolate was infected into a turbid suspension containing yeast extract 2 days prior to testing. 100 ml bacteria suspension was spread over the prepared agar plates. Using sterile tweezers, absorbent paper discs were dipped in actives and placed on the petri dishes as in figure 5. Petri dishes were sealed and incubated at 26°c for 48 hours before inspecting. Measurements were made of the diameter of inhibited bacteria growth around each of the paper discs and recorded. The test was repeated twice.

Watercress seed was mixed in a seed bubbler and a 4% solution of hydrogen peroxide was added to review its effect on bacterial loading of the seed. This was left for 15 minutes before draining. Samples of seed treated and untreated were sent to a lab for testing for presence of E coli and pseudomonas. E coli were tested for using a Chromogenic Coliform Agar where E. coli appears as metallic blue to violet colonies on the agar. Test was repeated weekly over 10 weeks. Data was uploaded onto the statistical package R and plotted on box plots. The nonparametric Wilcoxen rank sum test was carried out.

Water recirculating through the production units passes through an ultra violet system and we looked at its effect on E coli levels as well as *Xanthomonas spp*. Samples of water were taken immediately prior to and immediately after the UV unit and sent to Fera, UK for analysis. Samples were also taken weekly over 10 weeks and tested for levels of E coli at a lab in Seville. A paired t-test was carried out using the statistical package R to compare water samples pre and post treatment.

Results

Where healthy watercress plants were placed under plastic for 4 days, necrotic lesions on lower leaf margins were noted at the hydathodes. Samples of both leaves affected and healthy leaves were analysed in a lab for macro/ micro nutrient content and the results were compared (figure 6).

	Leaf margins affected	Healthy leaves	notes
N2 total %	**4,47**	**7,36**	
P %	**0,61**	**0,99**	
K %	**3,46**	**3,54**	
Ca %	3,79	1,85	
Mg mg/kg	0,52	0,35	
Mn mg/kg	236	143	
B mg7kg	42,3	22,1	

Figure 6: Macro/ Micro analysis of visibly healthy leaves against watercress leaves showing leaf edge necrosis.

As shown in figure 6, levels of N, P were lower in the leaves affected by senescence. Levels of Ca, Mg, Mn and B were higher in affected leaves. Level of K was similar in both healthy and unaffected leaves.

Following sequencing work at Fera and subsequently at The University of Warwick, it was confirmed that *Xanthomonas nasturtii* amongst other organisms was isolated from samples of watercress plant material (Appendix 2). At Warwick, the isolate from watercress crops in Spain

was noted as being different from the Florida strain, Vicente (2017) since it did not make the medium go brown. Xanthomonas nasturtii was also isolated from watercress seed produced at Royalcress in Spain (Appendix 3) and Xanthomonas spp. in water recycling through the production unit (Appendix 4).

The inoculation of watercress plants grown in Spain and at Warwick University with the isolate had two aims; to test for pathogenicity of *Xanthomonas nasturtii* on watercress and to look for differences in pathogenicity between plants treated with copper gluconate and those untreated. Crops analysed for copper content following treatment with the following results (figure 7) showing higher levels of copper in those plants treated;

	Applications	Level of copper (mg/kg)
RD16 treated with copper	2 applications of 6% copper gluconate applied radicular. Sample taken 48 hrs after first application.	16.4
RC 8 untreated	No treatment applied.	9.3

Figure 7: Copper content of treated/ untreated plants

(a)


(b)


(c)

(d)


(E)

Figure 8; 10 days following (A) wounding/ Inoculation of treated plant (B) wounding of treated plant (C) wounding/Inoculation of untreated plant (D) wounding of untreated plant (E) Symptoms seen after 10 days at Warwick University as reported by Vicente (2019).

Plotting % necrosis against treatment we see a clear difference in level of necrosis between the control plants (treated and wounded [B]/ untreated and wounded [D] and the plants inoculated (figures 8 and 9). Higher levels of necrosis noted on plants not treated with copper gluconate (figure 8 (c) [C] and inoculated against those plants treated and inoculated [A]. P value (aov) of 4.07 e-11 showed a significant difference between levels of necrosis and treatments.

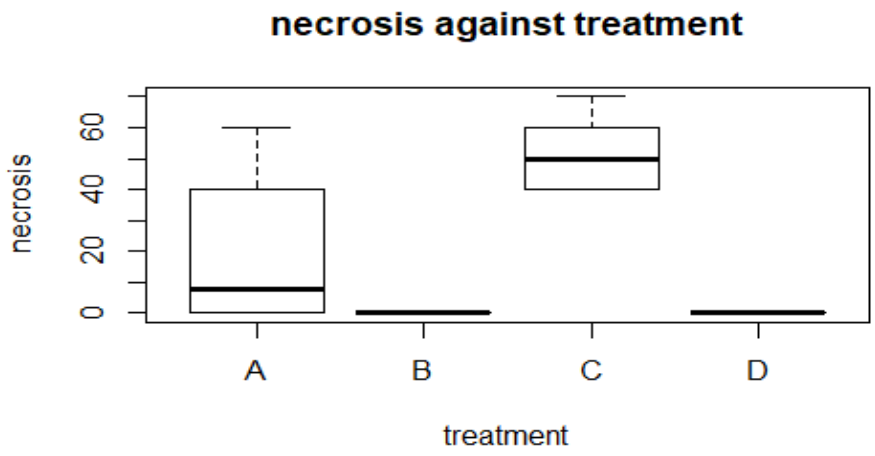


Figure 9; box plot showing treatment against level of necrosis;

Field trials.

Looking at effect of a number of organically approved products on disease levels in crop; where we saw a difference through the winter months was between crops treated with copper gluconate and the control within the same block of beds.

The first evidence of leaf edge senescence was noted in December on beds RD 13, 14, 15, 16 in the days following the harvest of beds RD4-12 inclusive (final harvest in these beds was 7/12/2017). Temperatures had dropped (Appendix 1) with frosts noted 4th, 5th, 6th, 7th and 8th December followed by a rise in temperature and rainfall on the 11th. Beds RD13, 14, 15, 16 had not been harvested and, as temperatures increased 9th-12th December and rainfall recorded, leaf edge senescence was noted on these crops.
Beds RD13, 14, 15 were harvested on the 15th showing a low level of leaf edge breakdown although not of a rejectable level. Bed RD16 (control) had a higher level of leaf edge cell breakdown noted as leaf edge necrotic patches on older and younger leaves and was not harvested with the other 3 beds. 4ml of rain was recorded on the 15th and quality of crop improved allowing harvest on the 17th. A higher level of leaf edge senescence was noted at harvest over the previous 3 beds.

The following harvests took place in January. The lead up to the harvest of beds RD4- 12 inclusive saw rainfall between the 6th and 9th and relatively mild min temperatures for January (mean of 5°c). Low level of leaf edge senescence was noted on older leaves but not sufficient to cause a rejection pre or post-harvest. In the lead up to the harvest of beds RD13-16, we saw two frosts on the 16th and the 18th together with some frost damage noted on the leaves.

Slightly higher levels of leaf edge senescence were noted on these beds and slightly higher again on the control bed (RD16) which was harvested 2 days later than the other beds.

The third harvest of beds RD4-12 inclusive took place in February (16th-23rd). There was little rain in the lead up to harvest and we saw 3 frosts. Levels of leaf edge senescence remained at 3 since this was very much evident in the lower canopy however the majority was avoided at harvest. Levels of leaf edge senescence rose late Feb / early March in the lead up to the harvest of beds RD 13-16 as temperatures both rose and rainfall was noted almost daily from the 24th February to the day of harvest (4th and 6th march 2018). Similar level of senescence noted in all 4 beds.
Following the third harvest, beds were cleaned out for re-drilling allowing crops to be available during the natural flowering period of the year. End of shelf life assessment (figure 10) shows 2 out of 3 treated crops at Amber (crop showing signs of breakdown) and the contro bed recorded as Red (higher level of breakdown).

Bed	Date harvested	Crop	Date assessed	Green/ orange/ red
RD13	15/12/2017	Watercress	26/12/2017	Amber
RD14	15/12/2017	Watercress	26/12/2017	Amber
RD15	15/12/2017	Watercress	26/12/2017	Red
RD16	17/12/2017	Watercress	28/12/2017	Red

Figure 10; Shelf life assessments (day 11 after harvest).

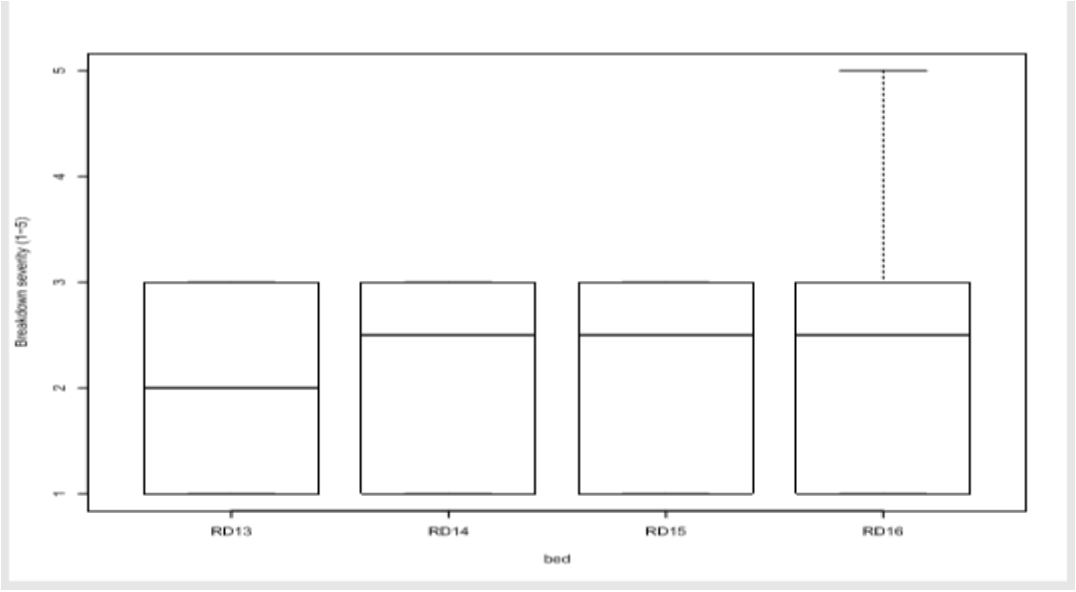


Figure 11- box plot showing bed against % breakdown severity.

A Kruskal- Wallis test gave a p value of 2.117e-07 showing a significance of date in determining severity of leaf edge senescence. A Friedman test gave a p value of 0.0293 showing a Significance of location (bed number) on leaf edge senescence.

In vitro efficacy testing of actives against bacteria.

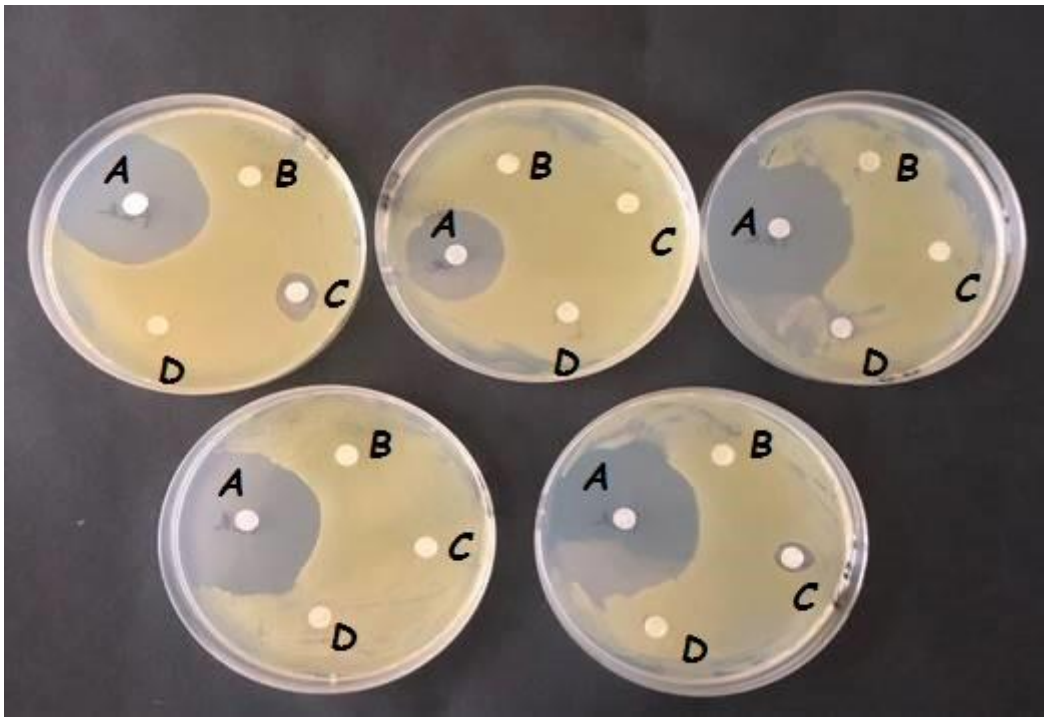


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
Hydrogen Peroxide (A)	2	1.5	2	2	2.5
Distilled wáter (B)	0	0	0	0	0
Copper Gluconata (c)	0.5	0	0.4	0	0
Bleach (D)	0	0	0	0	0.4

	Plate 6	Plate 7	Plate 8	Plate 9	Plate 10
Hydrogen Peroxide	1.5	1	1.6	2	2
Distilled wáter	0	0	0	0	0
Copper Gluconata	0	0.5	0.5	0.5	0.2
Bleach	0.5	1	2	2	1.5

Figure 11; Measurement (diameter) of circle inhibiting bacterial growth on plates.

Eficacy tests show a clear sterilant effect from hydrogen peroxide (figure 11) and a much smaller variable effect from copper gluconate. A clear antagonistic effect was noted on trials using Serrenade Max (Bacilus subtilis) can be seen in figure 12.

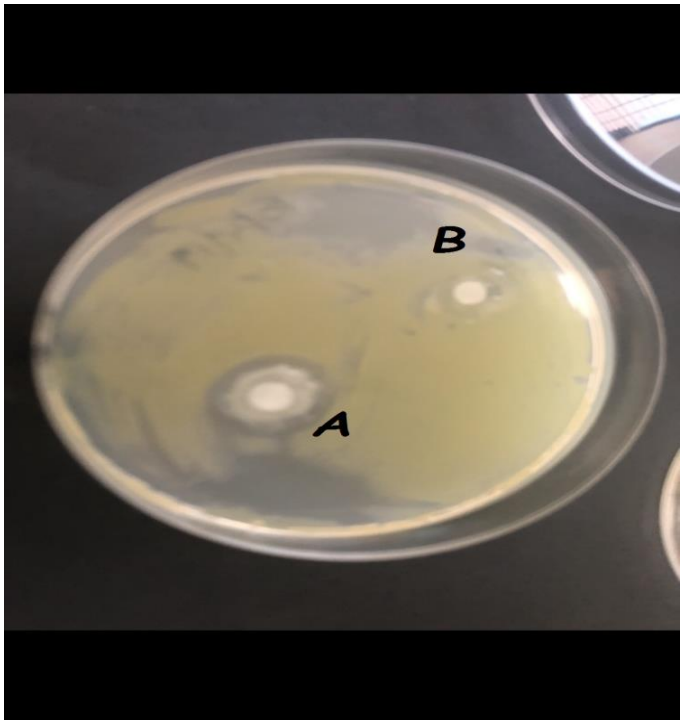
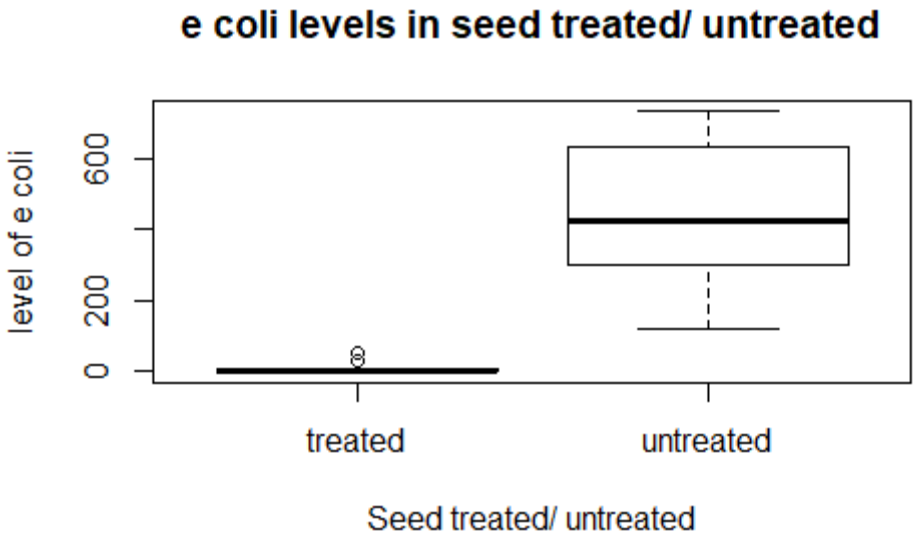


Figure 12: (A) antagonistic effect of Bacillus subtilis on Xanthomonas nasturtii (B) control; water.

Seed sterilisation.

Evidence here shows a clear reduction in E. coli and pseudomonas between batches treated with hydrogen peroxide and control.



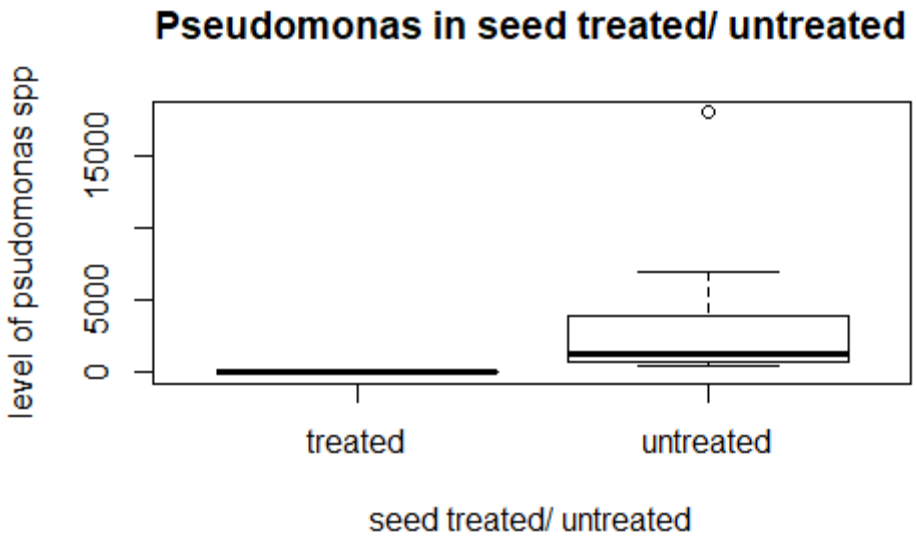


Figure 13: Effect of hydrogen peroxide on levels of E. coli and pseudomonas on watercress seed.

A Wilcoxon rank sum test with continuity correction was carried out on the data. A p value of 6.294e-05 indicated a clear reduction in E. coli and pseudomonas following treatment with hydrogen peroxide.

UV treatment of water recirculating through the watercress beds was assessed. Levels of e coli were evaluated in recirculating water before and after the UV unit. A paired t-test showed a reduction in E coli following UV treatment (p value 1.807e-05). Recirculation water screened at Fera pre and post UV showed no reduction in level of Xanthomonas spp (Appendix 4).

Discussion

Trials showed that raised humidity levels can induce leaf edge necrotic patches starting at the hydathode and this trial should be repeated to gain more confidence. During field trials symptoms were also seen during a period of elevated humidity following a period of low temperatures (appendix 1). Hugouvieux (1998) describes the hydathode as the entry point for *Xanthomonas campestris*. Since activity of bacteria is aided by increased humidity on the leaf whether it is through the use of sprinklers, McHugh and Constantinides (2004) or in this case increased humidity through covering crop with plastic, there is evidence that a pathogenic bacterium is causing the necrotic lesions.

Bacterial screening showed presence of *Xanthomonas nasturtii* on necrotic patches of watercress leaf material as well as on the surface of watercress seed. *Xanthomonas spp.* was identified in water recirculating through the production units. Presence of Xanthomonas in these lesions together with the fact that symptoms form under conditions such as increased humidity thus confirms that Xanthomonas is becoming pathogenic and causing the necrotic lesions. Identification of Xanthomonas nasturtii on watercress seed shows that this is seed borne and thus disease incidence reduction will necessitate identifying ways to eliminate the bacterium from seed.

The application of copper gluconate both in field trials and prior to the inoculation of *Xanthomonas nasturtii* isolate reduced the level of symptoms in watercress showing this active has potential for improving control in crops. Both foliar applications (during field trials) and radicular (prior to inoculation) proved positive and analysis of leaf material showed that, following radicular applications, copper gluconate had been assimilated by the plants (figure 7). The use of hydrogen peroxide had a marked effect *in vitro* on *Xanthomonas* although its effect might be short lived in field applications since its action is contact rather than being assimilated by the plant. Copper gluconate and Bacillus subtilis also showed an inhibitive effect on the growth of *Xanthomonas nasturtii in vitro* although that of copper gluconate was variable.

The use of hydrogen peroxide to reduce levels of *Xanthomonas in vitro* as well as E. coli on seed proved positive and trials should be repeated on see looking specifically at levels of *Xnthomonas nasturtii* on seed pre and post treatment. UV treatment of recirculating water decreased levels of E. coli although did not decrease levels of *Xanthomonas spp.* and further investigation and repetition of treatments is needed to understand why this is the case.

We see from field trials that symptoms appear when both environmental conditions and host (watercress) allow for pathogenicity to develop. In the case of field trials, mild humid weather followed a period of low temperatures which had resulted in damage to the crop. The disease triangle below illustrates the fact that, for a plant disease to develop, 3 main factors influence pathogenicity; susceptible host; presence of pathogen and favourable environment. In the field trial disease incidence came about when these three factors allowed it to do so.

In order to control *Xanthomonas* nasturtii, we need to manipulate these 3 factors and improvements in control are likely to result from small changes to all three.

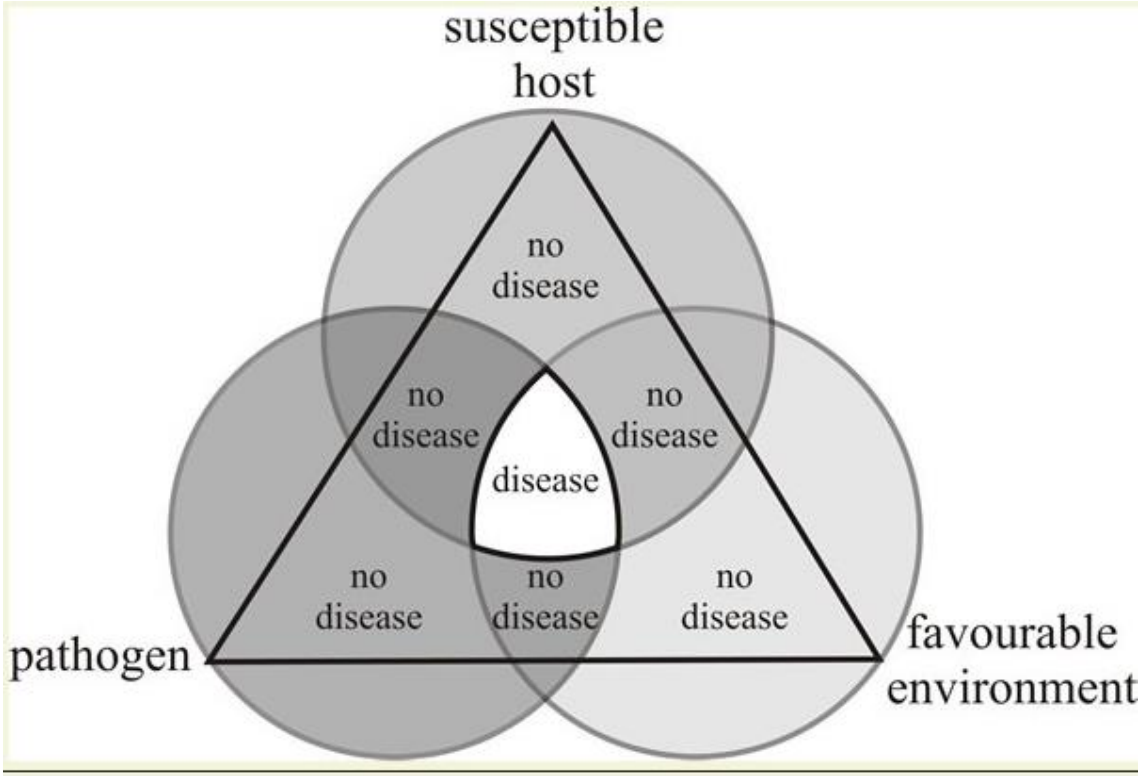


Figure 14: disease triangle, Moore et al (2018)

Manipulating environment.

It was noted that symptoms from which *Xanthomonas nasturtii* was identified could be induced through manipulation of the growing environment (humidity). One key factor that could have been affecting the above analysis was where the symptoms were seen. Symptoms such as in the photograph above (figure 8) were mainly seen on lower older leaves. Nutrients such as N, P, appear to be already being transported to healthy leaves situated higher in the canopy (younger leaves). Elements such as Ca are more heavily present in the older leaves affected are not being transported as quickly to the younger leaves due to lack of transpiration. However, elements being transported to younger leaves will weaken older leaves making them more susceptible to infection.

Manipulating the environment in field grown crops is the hardest of the three elements of the disease triangle to achieve. The weather data in the period when symptoms were seen were in a period of wet mild weather following a period of cold weather with frosty mornings and frost damage had been noted prior to symptoms appearing. However, some cultural methods of production can favour disease spread and pathogenicity. McHugh and Constantinides (2004) review *Xanthomonas* in Hawaii suggesting that overhead irrigation can favour *Xanthomonas* spread through splash as well as provide a humid environment which favours *Xanthomonas* development. Watercress is grown in fairly dense environments which can help raise humidity at lower canopy level. Reduction in density at drilling can be reviewed against acceptable yield.

Manipulation of pathogen.

Manipulation of the pathogen starts with timely identification and Roberts (2017); Billing (1987) stresses the need for growers to strive to understand bacterial diseases and their epidemiology in order to gain a deeper understanding where opportunities for improved control lie. He stresses early identification is essential in order to gain control rather than waiting until crop protection products fail. Here we see *Xanthomonas nasturtii* being identified by various institutions available using PCR allowing relatively quick multiplication and identification through extracted DNA.

Identification of source of infection is vital and we see here that seed is a source. Roberts (1999) describes *Xanthomonas campestris* as being seed-borne but also lists crop debris, weeds and soil as potential sources. We see that the water recirculating through the watercress production systems as well as seed can be contaminated with *Xanthomonas*.

With identification and an understanding of the environmental conditions favouring pathogenicity, vigilance is important. We saw in field trials symptoms appearing from December through to February when days are shorter; rainfall is higher as is incidence of low temperatures. Treatments which therefore aim to improve the plants own defences need to be applied prophylactically in order to build up plants defences and reduce disease incidence.

Roberts (2017) looks at control methods for a number of bacterial diseases in the UK. He points out that there have been no major new actives put forward in the last 20 years to control bacterial diseases and this is likely to remain the same for the foreseeable future. This may be due to the fact that most of the focus has been on diseases caused by fungi, Roberts (2017). Restrictions on use of actives known for good control of bacterial infections such as copper Oxychloride will increase. In Spain this active is no longer registered for use on watercress crops. Instead, growers should look at non chemical preventative measures in order to control bacterial infections. One of the main areas where control can be enhanced (also see disease triangle, fig 2) is through knowledge of both the bacteria but also the source of infection. We see that, within our watercress production system, *Xanthomonas* is seed borne and therefore improved control would be gained through use of clean seed. Seed used in the watercress production areas in Spain originates from plants grown in Spain.

U.V treatment of seed during the cleaning and drying stage may assist in reducing Xanthomonas levels at source. Kessel, G J T (2008) found that the bacteria Xanthomonas fragariae is very sensitive to UV. *Xanthomonas* spp. in recirculated water was unaffected by UV and further work is needed to understand why.

Roberts (2017) mentions use of sterilants their success in vitro and their lack of persistence in field trials. (HDC trials). We have seen in the efficacy tests a marked response to the addition of Hydrogen Peroxide. It would be worth noting from our tests and these comments the potential for use as a cleaning agent for machinery as well as seed although its use as a crop treatment may be limited. Since watercress seed is drilled dry, use of any liquid sterilant will have its difficulties. Heat readily kills bacteria and is used as hot water treatment of seed Roberts J (2013). Roberts (1999) demonstrated that tools such as secateurs could inoculate

significant number of plants following contamination raising the importance of effective sterilising programs for machinery coming into contact with crop.

The use of biological control agents has been increasing over the last 20 years with antagonistic non-pathogenic bacteria such as bacillus subtilis being used to suppress bacterial diseases, Roberts, J (2017). In our efficacy tests we see bacillus subtilis suppressing *Xanthomonas nasturtii* development. Products such as Serrenade max (a powdered preparation) may be used as a seed inoculant. Chen et al, (2013) describe the use of Bacillus subtilis in the control of the plant disease causing bacteria *R Solanacearum*. They point out the need for positive antagonistic behaviour and strong biofilm formation of the bacillus subtilis strain in vitro in the selection of suitable biocontrol agents. We have seen strong biofilm formation in vitro of bacillus subtilis and an indication of an antagonistic action. Roberts (2017) reported mixed results using Serrenade in the control of bacterial pathogens and it would be useful to undertake more trials on plants in the field as well as in a controlled environment to understand potentials for its use on watercress.

Phages (viruses which target bacteria) are another option for potential and, in fact, early phages discovered targeted black rot (*Xanthomonas campestris*) using a filtrate of cabbage showing disease infection, Holtappels (2019). Recent trials using bacteriophage to control *Xanthomonas euvesicatoria* on peppers in Serbia has proved positive, Sevic et al (2019). These are natural enemies of bacteria and specific to a particular strain rather than broad ranged as are antibiotics. They are self-replicating and can adapt as the bacteria do to resist treatments making them more sustainable. This makes them ideal in one sense that they attack only the pathogen rather than any beneficial bacteria but a disadvantage in that each phage product developed will only target a specific strain thus increasing costs of development. Targeting specific strains also reduces interest in developing products targeting crops such as watercress where total area cultivated may well not bring sufficient return on investment.

Roberts (2017) talks about the use of Elicitors. Elicitors are compounds who control disease by switching on or enhancing the plants innate defences against pathogen attack resulting from a greater knowledge on host-pathogen interaction plus biochemical pathways. Roberts (2017) suggests this is at research stage and has potentials for future control.

AMBER, an initiative lead by The University of Warwick, UK looks to help growers optimise the use of biopesticides. Biopesticides generally require an in depth knowledge by the grower on their use and the environmental conditions necessary to be effective. Amber assists through research initiatives and knowledge transfer. It may be that a combination of biopesticides and a reduced input of actives such as copper gluconate would produce positive results and the use of such advice centres can only be useful.

Removal of pathogen and avoidance of carry over between crops can take a number of forms. Meenu, G et al, (2013) describes *Xanthomonas* as surviving on infected plant material. Watercress is a crop which, during the winter months, is subjected to multiple harvests. Between each harvest, the crop is topped off with a mower to ensure even growth through removing excess plant material at the top of the stubble (post-harvest). This material may well harbour diseases such as *Xanthomonas* and modification of the topping procedure to take

away this excess material will be positive. In the summer when watercress is not produced on the Spanish farms, beds are cleaned out to ensure no carry over of crop debris occurs from one season to another.

Seed is produced in Spain for supply to farms belonging to the watercress Company in the UK, Spain and Florida. The majority of commercial seed is tested for presence of bacteria. However, since watercress seed tends to be produced on farm by growers, reducing the bacterial loading on seed harvested in Spain should be a primary focus in reducing the source of *Xanthomonas*. Meenu, G (3013) talks about carryover of Xanthomonas campestri bacteria on seed of cauliflower and other crucifers as a survival mechanism/ reinfection surviving for up to 3 years.

Various ways of reducing bacterial loading on seed produced in Spain could include;

- Hygiene in the field. Overhead irrigation can help spread bacteria, Al-Saleh, (2011). Automatic irrigation early morning allowing crop to dry out in day to avoid development of bacterial diseases. Alternative irrigation systems such as drip could be trialled.
- Treating the seed crop during its growth stage avoiding a build-up of bacteria on and in the plants.
- Avoiding bacteria bridging from one seed crop to another through debris removal, soil treatment. Crop rotation can be options but lifecycle and understanding how long the bacteria can survive in soil needs investigating first. Bayer on their website list rotation as a control for Xanthomonas campestri (black rot).

Manipulating host

Inducing resistance in the host is an option and Gonzalez (2018) reports the use of copper gluconate inducing the accumulation of phenolic compounds. He also reported a reduction in reactive oxygen species (ROS which is involved in the stimulation of hypersensitive cell death, a response to attack). However the build-up of ROS can lead to oxidative damage in membrane lipids, proteins and nucleic acids.). This suggests that has an effect on pathogenesis machinery or induces defences. To see if copper gluconate induces plants defences, Gonzalez measured phenolic compounds that have both a direct antimicrobial activity as well as inactivating microbe-produced enzymes involved in pathogenesis or inhibit synthesis of specific toxins. He reported accumulation of phenolic compounds so suggesting induction of plants defences. Reduction prevents bacteria reaching populations where pathogenicity occurs (quorum sensing).

Protecting the plant from infection plays an important role in crop protection. Providing optimum nutrients without weakening the plant through applying excess will maintain vigor and aid in defense. Protecting plants from the wind driven rain involves vigilance on weather forecasts and covering crops with hail mesh in the event of a weather event potentially damaging the crop. Use of frost protecting fleece (30g specification fleece) at night during periods of frost risk reduces potential damage to the crop.

Resistance to bacterial infections is one way of improving control. Improved understanding of the host/ bacterial interactions could result in developments in marker assisted selection as part of plant breeding. Introduction of transgenic resistance is another. There has been little work to date in this area where watercress is concerned. However Payne et al (2015) looked at increasing knowledge of variation occurring across the watercress genome potentially allowing for further research looking at particular desirable traits evident in both cultivated and wild watercress populations. Next generation sequencing is making this process a lot more achievable both in terms of cost as well as time, Payne et al, (2015).

Scope for further work.

Further work understanding the strain of *Xanthomonas nasturtii* and potential differences with the strain found on watercress in Florida might reveal some clues as to its development and potential control. Pathogenicity tests using seed kept at gene banks such as Wellesbourne may highlight useful traits amongst accessions that could be used in breeding programs to improve resistance.

Trials increasing humidity should be carried out to gain more understanding of the role of humidity in symptom development.

Further understanding of the natural defence systems of watercress would assist in understanding of how new products marketed to assist in the plants defences can be more efficiently used. Measuring the effect on the plant of applications of copper gluconate would establish routes of resistance activated or enhanced through such treatments.

The use of bacillus subtilis in vitro showed promise and it would be useful to carry out trials in controlled atmosphere on plants as well as in field. Seeking off label approval for its use in Spain would facilitate these trials.

Further work on how UV affects Xanthomonas nasturtii is needed in order to understand whether this might be used as a control option for Xanthomonas in recirculated water.

Acknowledgments; Thank you to my supervisor Dr Michael Roberts at Lancaster University. Thank you to Joana Vicente who isolated *Xanthomonas nasturtii* from samples of watercress grown in Spain. Thanks also to Andrea Azpilicueta (Cambrico Biotech, Seville) who enabled me to carry out in vitro tests as well as prepare bacteria solutions for and gain practical experience inoculating watercress plants with *Xanthomonas nasturtii* isolated from plants in Spain by Joana Vicente.

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Appendices;

Max Min Temperatures September 17 - Feb 18.

Date			*Min*			
	Sep	**Oct**	***Nov***	***Dec***	**Jan**	**Feb**
1	19	15	*13*	*3*	5	4
2	21	17	*17*	*2*	3	2
3	20	16	*16*	*2*	7	0
4	16	17	*16*	*1*	8	2
5	18	12	*10*	*0*	12	0
6	19	14	*11*	*0*	6	2
7	18	13	*7*	*1*	2	-1
8	17	14	*6*	*1*	3	2
9	13	13	*10*	*4*	2	0
10	13	15	*7*	*8*	8	2
11	13	12	*5*	*7*	4	**1**
12	16	19	*5*	*5*	3	4
13	15	18	*5*	*2*	4	1
14	15	15	*6*	*2*	3	1
15	16	16	*4*	*9*	3	2
16	9	18	*5*	*5*	**1**	3
17	11	21	*5*	*3*	6	7
18	9	17	*6*	*1*	**2**	4
19	11	16	*5*	*4*	5	4
20	13	15	*3*	5	4	3
21	13	17	*2*	*3*	5	5
22	14	17	*3*	3	4	2
23	16	14	*5*	2	2	0
24	15	13	*6*	3	3	3
25	14	10	*5*	4	8	6
26	12	11	*12*	8	4	4
27	14	11	*9*	12	**2**	12
28	17	11	*12*	10	3	14
29	15	10	*10*	11	8	
30	15	11	*5*	9	10	
31		11		9	12	
Averages	***15***	***14***	***8***	***4***	***5***	***3***

Max Min Temperatures September 17 - Feb 2018

Date			Max			
	Sep	Oct	Nov	Dec	Jan	Feb
1	36	34	26	14	17	17
2	35	34	25	13	17	13
3	36	33	23	11	19	14
4	33	33	22	15	16	12
5	34	35	21	18	18	13
6	38	32	22	16	14	12
7	37	33	21	18	12	13
8	37	34	22	17	11	14
9	28	35	19	18	14	13
10	29	33	19	19	17	15
11	36	31	23	17	16	17
12	38	34	23	13	13	17
13	37	36	19	15	14	15
14	32	35	18	17	13	17
15	26	34	22	19	13	21
16	25	29	21	15	17	22
17	27	29	22	14	17	19
18	29	22	21	15	20	19
19	31	23	23	17	18	19
20	33	26	23	16	19	22
21	32	28	25	17	16	19
22	32	28	24	18	19	18
23	33	28	25	19	18	19
24	33	29	27	16	17	16
25	33	29	24	16	17	17
26	30	30	23	16	14	22
27	26	30	23	17	15	19
28	30	29	21	18	16	16
29	33	27	20	19	18	
30	33	26	15	21	17	
31		27		18	19	
Averages	**32**	**31**	**22**	**17**	**16**	**17**

RSA Max Min Temperatures September 17 - April 18

Date	***Rainfall***					
	Sep	**Oct**	***Nov***	***Dec***	**Jan**	**Feb**
1	0	0	*0*	0	0	0
2	0	0	*0*	0	0	0
3	0	0	*16*	0	0	0
4	0	0	*0*	0	0	**7**
5	0	0	*0*	0	0	0
6	0	0	*0*	0	24	0
7	0	0	*0*	0	**3**	0
8	0	0	*0*	0	1	0
9	0	0	*0*	0	8	0
10	0	0	*0*	0	0	0
11	0	0	*0*	17	0	**0**
12	0	0	*0*	0	0	1
13	0	0	*0*	0	7	0
14	0	0	*0*	0	**2**	0
15	0	0	*0*	4	0	0
16	0	0	*0*	0	0	0
17	0	60	*0*	0	0	0
18	0	33	*0*	0	0	**0**
19	0	0	*0*	0	0	0
20	0	0	*0*	0	0	0
21	0	0	*0*	0	**0**	0
22	0	0	*0*	0	0	0
23	0	0	*0*	0	0	0
24	0	0	*0*	0	0	0
25	0	0	*0*	16	6	**0**
26	0	0	*0*	1	0	7
27	0	0	*0*	1	0	5
28	0	0	*1*	0	**0**	15
29	0	0	*40*	0	0	
30	0	0	*0*	0	0	
31		0		0	0	
Averages	**0**	**93**	***57***	***39***	**51**	**35**

Appendix 1; Temperatures max and min during field trials.

Appendix 2; identification of Xanthomonas nasturtii on watercress 2017/ 2018.

Customer:	Royalcress SA
E-mail:	damien.lascelles@royalcress.com
Customer Ref:	watercress pseud001
Fera Reference:	21723449
Sample Received:	21/12/2017
Date:	17/01/2018

Final Report for : Sample of Nasturtium officinale

We have completed all tests and found the following.

Sample **21723449** : Results from **Bacteriology** department:
Sample Received: 21/12/2017
Customer ref: watercress pseud001
Host: Nasturtium officinale

Xanthomonas nasturtii

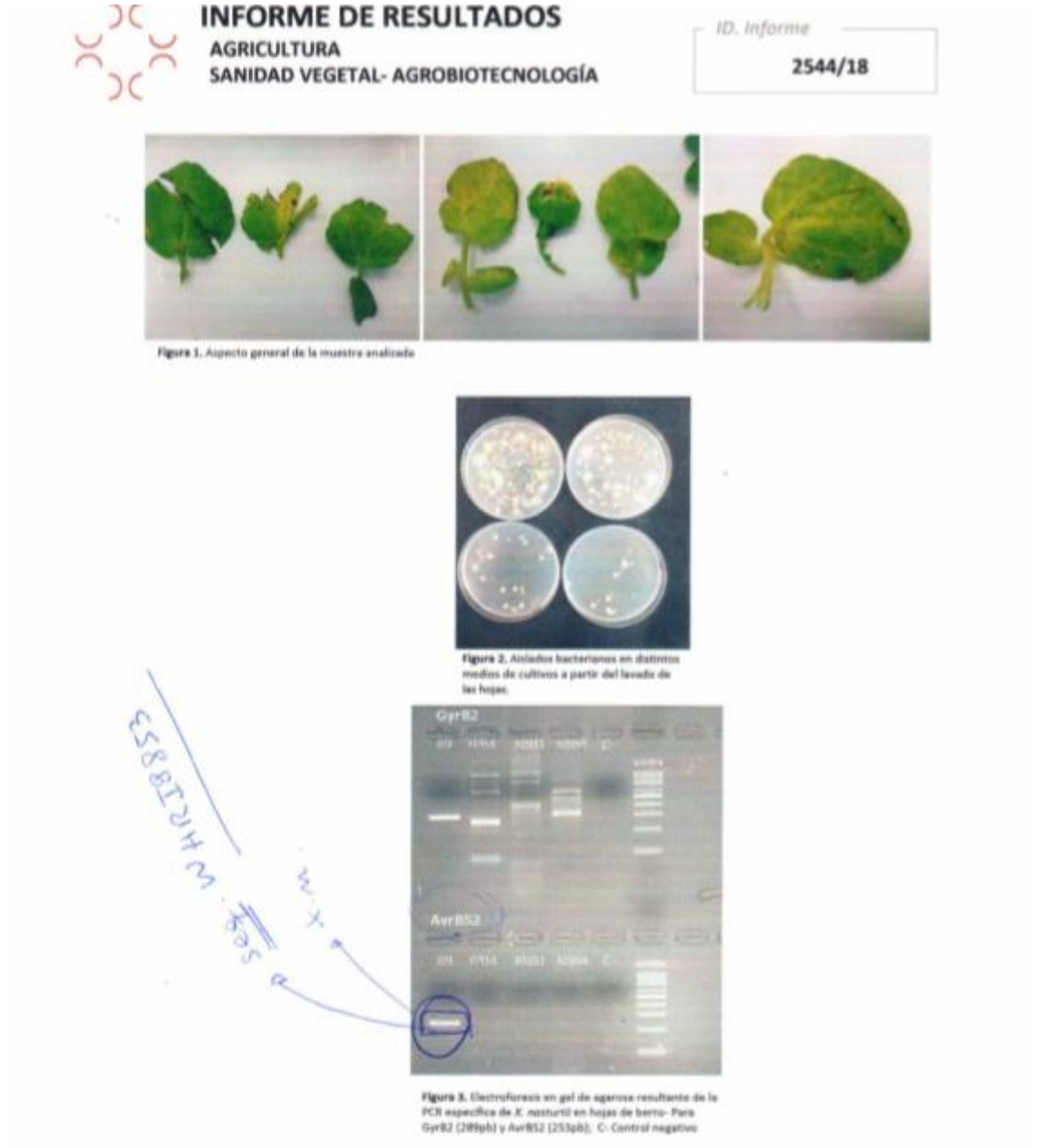
A *Xanthomonas* spp. was isolated from the material we received and characterised using fatty acid profiling. Partial gene (gyrB) sequencing was carried out, the gyrB profile generated matched that of *Xanthomonas nasturtii*.

I hope this information is of use to you. If you have any queries regarding this report, please contact me on the number below.

Yours sincerely,

Adam Bryning
Plant Bacteriology Diagnostician

For general enquiries about your sample or our services please contact Plant Clinic on:
Tel: +44 (0)1904 462 324 | **E-mail** plantclinic@fera.co.uk | **Web:** www.fera.co.uk/plantClinic
Post: Fera Science Ltd. (Fera), Sand Hutton, York, YO41 1LZ U.K.


INFORME DE RESULTADOS
AGRICULTURA
SANIDAD VEGETAL- AGROBIOTECNOLOGÍA

ID. Informe
2544/18

Figura 1. Aspecto general de la muestra analizada

Figura 2. Aislados bacterianos en distintos medios de cultivos a partir del lavado de las hojas.

Figura 3. Electroforesis en gel de agarosa resultante de la PCR específica de *X. vesicatoria* en hojas de berro- Para GyrB2 (289pb) y AvrBS2 (253pb); C- Control negativo

Identification of Xanthomonas in Spain (Cambrico Biotech, Seville).

Appendix 3: Identification of Xanthomonas on watercress seed in Spain 2019.

Customer: Damien Lascelles
Royalcress SA
E-mail: damien.lascelles@royalcress.com
Customer Ref: watercresseed2019
Fera Reference: 21902792
Sample Received: 15/02/2019
Date: 06/03/2019

Final report from Fera Plant Clinic: Bacteriology

Final report for Nasturtium seed sample

This is the final report from the Bacteriology team, and this completes the work by the team.

Our ref	Your ref	Result	Our comment
21902792	watercesseed2019	Xanthomonas sp.	The 10,000 Nasturtium seed sample was tested for the presence of Xanthomonas nasturii using methods based on the ISTA validated method for Xanthomonas campestris pv.campestris on Brassica seed.

We isolated a Xanthomonas sp. from the seed sample provided. XgyrB partial gene sequencing was carried out which gave a 99% match to the Xanthomonas nasturii type strain.

I hope this information is of use to you. If you have any queries regarding this report, please contact me on the number below.

Once testing is complete, we dispose of samples within 10 working days

Yours sincerely,

Adam Bryning

Diagnostician

Appendix 4; Identification of Xanthomonas spp. in water recirculating through watercress beds, Spain.

Final report from Fera Plant Clinic: Bacteriology

Pre and Post UV Treatment Water Samples

This is the final report from the Bacteriology team, and this completes the work by the team.

The two water samples provided (Pre-UV Treatment and Post UV Treatment) were tested for the presence of Xanthomonas spp.. A total viable count was also completed as requested.

Our ref	Your ref	Result	UKAS accredited	Our comment
21910584	Pre & Post UV	*Xanthomonas spp.*	Yes	**Post-UV Treatment** A total viable count (TVC) of 250,000 cfu/ml was achieved following isolation on Nutrient agar medium. An approximate count of 1,100 cfu/ml of *Xanthomonas* spp. was achieved following isolation on a range of appropriate non and semi-selective media.
		Xanthomonas spp.	Yes	**Pre-UV Treatment** A total viable count (TVC) of 290,000 cfu/ml was achieved following isolation on Nutrient agar medium. An approximate count of 1,200 cfu/ml of *Xanthomonas* spp. was achieved following isolation on a range of appropriate non and semi-selective media.

Identification of isolated *Xanthomonas* spp. was completed using Fatty Acid Profile analysis of isolates exhibiting typical colony morphology in accordance with methods accredited to ISO17025 - PLH/018, PLH/019 and PLH/020.