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TEST PLAN AND PROCEDURES

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1. Introduction

1.1. Choice of crop and cultivar

The proposal of this project proposed the use of lettuce or beet depending on the results of WP2000. This was based on the experience gained on these crops within the MELiSSA consortium by the research group in Guelph (Cloutier and Dixon 1997; Cloutier and Dixon 1998; Masot et al. 2005; Masot et al. 2004). Taking into account the obtained data overview within WP2000 (see TN89.11 and TN89.12) the best crop choice would be lettuce because more literature (which can function as relevant reference material) on hydroponic growth is available for lettuce compared to nearly none for beet. Since the proposal stated to use the same cultivar as used in the research group in Guelph, it will be: *Lactuca sativa* L. cv. Grand Rapids.

1.2. Choice of stresses

The proposal stated to test two stresses which were classified as one of the most critical ones in TN89.12. From the 14 stresses listed in Table 1 in TN89.12 most stresses are very critical and will need to be paid attention to. Ranking the stresses by taking into account the 3 defined parameters (importance, chance of failure, impact) with equal weight can give estimation, but it is important to reiterate the fact that limited experimental data is available to support this exercise as a finalized criticality ranking. Most notably the impact of a stress will differ depending on the growing conditions of the plant, hence comparison among studies with different conditions and setups can only be indicative op possible impact. Moreover such published research conducted in controlled conditions is very limited in number.

Table 1: Preliminary stress factor ranking

Stress	Ranking	Stress	Ranking
Light	9	Root-oxygen	6
Temperature	8	Nutrient levels in general	6
CO_2-level	7	Nutrient level - Ca	6
Root zone water	7	Bacterial pathogens	5
Ethylene	7	Air humidity	4
Fungal pathogens	7	Viral pathogens	3
Pathogens in general	7	Allelopathic factors in general	3

Optimal light, temperature and CO_2-level are the first requirements for high crop yields. However the effect of deviations of these 3 parameters from optimal values (which are exclusively due to system breakdowns) is well-known, and should be minimised in time by appropriate alarm systems and system redundancy.
The even short time absence of root-zone water in a hydroponic (NFT) system will lead to irreversible damage and crop loss. The same approach should be followed as for the 3 above-mentioned parameters to avoid the occurrence of this stress-situation.

Next in the ranking are ethylene accumulation and fungal pathogens. The middle to-long term (visual crop growth inhibition) effects of these stresses are to a large extent characterised. Fungal damage is ubiquitous in agriculture, and the effects of ethylene on plant growth in closed spaces has been clearly observed during previous studies (see TN 89.11). However, the aim of this project is to minimize the impact of stresses by early pre-visual detection and associated corrective action, to avoid negative effects on crop yield.
Nowadays, destructive sampling of supposedly (based on visual indications) pathogen-infected plant tissue permits the determination of the causal agent (fungal or bacterial) by commercial molecular-genetic testing for approximately 40 pathogenic organisms in parallel (DNA amplification and hybridisation - http://www.dnamultiscan.com). This approach is also used for a periodic (weekly to monthly) check-up for pathogen presence in the hydroponic nutrient solutions used in commercial greenhouses.
The ability to detect the earliest sign's of plants stress non-destructively holds promise to use this information for site-directed sampling, causal agent identification and site-specific treatment or sanitation, with minimal effects on final crop yield.
Among pathogens, fungi are known to spread rapidly via airborne spores produced on aboveground plant parts. Moreover nutrient solution borne oömycete (water moulds) infection can cause considerable losses. Among the oömycete plant root pathogens, *Pythium* species are reported to be a major problem in hydroponic cultures (Al-Saadi et al. 2007; Herrero et al. 2003). Although such an infection cannot be excluded in a growth room-based setup, its chance of occurrence will certainly be lower than ethylene accumulation, although impact on crop yield could be more devastating.
Ethylene accumulation is acknowledged as a major problem specific for closed environmental growth systems. In a long-term crop growth trial ethylene accumulation will occur, the effects will however depend on crop species and cultivar. Therefore early monitoring and quantifying of crop growth and general physiology will allow defining the needed countermeasures.

For the proposed one crop-one stress study ethylene accumulation stress in lettuce will be tested by ACC addition to the hydroponic solution. *Pythium* root infection could be tested in a future follow-up study.

1.2.1. Ethylene

To study the effects of ethylene it can be applied directly as a gas to the atmosphere, or it can be supplied as ACC (1-aminocyclopropane-1-carboxylic acid) to the nutrient solution (Smalle and Van der Straeten 1997). ACC is the immediate precursor of ethylene, which is easily taken up by the roots and transported through the plant. The plant will convert it to ethylene, which consequently will have an effect on the plant's growth and development, mimicking the effect of an increased air ethylene level. When using ACC as the stressing agent instead of ethylene gas, control plants can be grown parallel with the treated plants in the same growth chamber, and within the same experimental crop cycle. This will save time and resources compared to the required serial runs for treatment and control for ethylene gas treatments.

First, a preliminary crop growth experiment will be carried out, during which lettuce will be grown under control conditions in both independently controlled hydroponic circuits. Growth kinetics will be determined based on the evolution of projected leaf area from the imaging sensors, and will be complemented by other online and offline measurements.

In the subsequent stress treatment experiment, ACC will be added to the feeding tank of the "treatment" hydroponic circuit when the plants are 14days old, to reach a 10 µM final concentration. This will allow to monitor the plants individually for one week before the treatment, as to obtain a reference starting value on plant size. Older plants will in general be less sensitive to the treatment. ACC will be taken up by the roots, and ethyelen will be produced in the plant where it will exert its growth inhibitory effects. Comparison of the growth dynamics between the "Treatment" and "Control" plants, and additionally with the 'reference' kinetics obtained during the preliminary crop growth experiment will indicate the magnitude of the inhibitory ethylene effect. The results of the on-line thermal and chlorophyll fluorescence imaging will be compared between control and treatment to determine the ability to detect early signs of the induced stress. To determine if the ACC root treatment will induce ethylene accumulation in the gas phase (non sealed chamber atmosphere) to a level that could affect the parallel "Control" treatment plants, online measurements will be carried out in parallel to monitor chamber ethylene levels. Depending on the obtained results, the ACC concentration to be used in the subsequent experiments will be adjusted.

1.2.1.1. Ethylene treatment protocols

Required amount of ethylene:

Gas bottle:	50 L at 150 bar = 7500 L at 1 bar
	1000 ppm ethylene
Fresh air supply rate:	0 to 2 m^3 h^{-1} (2000 L h^{-1})
Ethylene supply to maintain 1 ppm:	0 to 2 L h^{-1}
At maximum air refreshment rate and continuously supplied:	
One bottle lasts:	7500/ 2 = 3750 h = 156 days = 22 weeks
Chamber volume:	3.15 m (l) x 1.5 m (w) x 2.15 m (h) = 10.16 m^3
Volume air-circulation unit + ducts:	5.31 m^3
Equilibration time:	for 10.16 + 5.31 = 15.47 m^3 total volume
	15.47 m^3/ 2 L h^{-1} = 7.74 h

Required amount of ACC:

ACPC (C4H7NO2) ≥ 95 % FLUKA	
Max growth area:	4.5 m^2
Area treated:	2.25 m^2
Volume nutrients/ area treated:	40 L
Plant water uptake:	1.46 L m^2 d^{-1} (Masot *et al*., 2004; 24 h light d^{-1})
(measured as produced condense water)	
Mol. Weight:	101.1 g/mol
Treatment concentration:	10 µM (1.011 mg/l)
Initially required:	40.44 mg
Required to maintain 10 µM:	2.25 x 1.46 x 1.011 = 3.32 mg d^{-1}

1.2.2. Pythium

There are over 80 plant pathogenic *Pythium* species, which in general have a broad host range (Kong et al. 2004). Their abundance and pathogenicity varies widely (Al-Saadi et al. 2007; Herrero et al. 2003). One of the most common and at the same time most pathogenic strains is *Pythium aphanidermatum.* Lettuce is major host for *Pythium aphanidermatum* (Crop Protection Compendium; http://www.cabicompendium.org). Therefore *Pythium aphanidermatum* would be the inoculum of choice for the stress experiments.

1.2.2.1. Pythium inoculation protocols

A Pythium inoculation protocol was described in Johnstone *et al*. (2005). Zoospores of *P. aphanidermatum* isolate P6 for inoculation of hydroponic bell pepper were produced as follows. The isolate was grown on V8-juice agar medium in petri dishes at 25°C for 48 h, after which the medium was cut into six 1-cm-wide strips. Alternate strips in each dish were transferred to an empty petri dish, and all dishes were flooded with 25 ml of sterile distilled water. The plates were incubated at 25°C under fluorescent lights (needed to induce zoospore production), and after 48 h, the water was replaced with another 25 ml of sterile distilled water. After incubation at 20°C under fluorescent lighting for 4 h to stimulate zoospore release, zoospore suspensions were collected and zoospore density was estimated with a hemacytometer. Plants were inoculated 14 days after transplanting by immersing the root systems in suspensions of 6.25×10^3 zoospores per ml of sterile water for 30 min. Roots of control plants were immersed in sterile water for 30 min. (Johnstone et al. 2005).
An alternative Pythium inoculation protocol was used by Herrero *et al*. (2003) and Al-Saadi *et al*. (2006). These authors did severity checks of several isolates of Pythium on cucumber seedlings (in soil mixture). Agar plugs of 10 mm diameter from 48-h cultures grown on PDA (potato dextrose agar) dishes were used for the inoculation. Four plugs were used per pot; each plug was placed near a seedling avoiding direct contact. The plants were irrigated daily with tap water. After 6 days the plants were gently pulled out of the pots and the roots were washed to observe the damage. The number of dead and damaged plants was noted without making any further gradation of symptoms. (Al-Saadi et al. 2007; Herrero et al. 2003)

1.3. Plant density and area

Culture conditions will as much as possible be adapted to the conditions used in at UoG. For NFT (Nutrient Film Technique) grown lettuce a density of 24 plants m^{-2} was used in the Guelph setup. We will use a plant and gully distance of 20 cm to maintain this density. The climate chamber contains 6 shelves of 1.6 m length and 0.51 m width. Each shelf is equipped with two gullies of 1.5 m length and each gully will hold 7 plants with a plant distance of 20 cm.

1.4. Culture conditions

Conditions at UoG (NFT grown lettuce in 2004, (Masot et al. 2005) were as follows:

Germination on Rockwool cubes (1.5 square inches, 9.4 cm^3) in the greenhouse. Plants were grown for about 20 days before being planted in the growth chamber NFT system. During these 20 days, the plants were regularly watered with distilled water and weekly with fertilizer solution (20-8-20 N-P-K commercial mix having an EC = 2.5 dS m^{-1}. Thereafter the plants were transferred: the small Rockwool cubes were placed in larger Rockwool cubes (4 x 4 x 2.5 square inches, 625 cm^3). Lettuce plants were grown for about 35 days in the growth chamber (total growth period from germination about 55 days).

Light	400-450 µmol m^{-2} s^{-1} PAR at stand height
Photoperiod	14/10 hours light/dark
Temperature	26/ 20 °C day/ night
CO_2 concentration	1000 µL L^{-1}
Humidity	73% ±5 %

1.4.1. UG-HSB Experimental conditions

The following table summarizes the conditions in the measurement chamber.

Table 2: Conditions to be used

Light	250-400 µmol m^{-2} s^{-1}
Photoperiod	24 h light
Integrated PPF	34.56 mol m^{-2} d^{-1}
Temperature	25 ºC
CO_2 concentration	ambient
Humidity	75%
Airflow*	0.35 m s^{-1}
Air refreshment rate	Max. 2 m^3 h^{-1} 0.027%
Plant density	24 m^{-2}
pH nutrient solution	5.5-5.8
EC nutrient solution	0.5-0.6 dS/m germination stage
	0.5-1 dS/m seedling stage
	2-2.5 dS/m vegetative stage

*The air entry wall measures 2x3m. The open area of this perforated wall is 35.4 % (Fig. 1). Standard flow rate of the ventilation system: 7500 m^3 h^{-1}. Generated flow at the entry wall: 0.98 m s^{-1}, and in the chamber: 0.35 m s^{-1}.

Figure 1: Calculation of growth chamber air speed

Calculation of open area: round holes, triangular pitch

$$\frac{R^2 \times 90{,}69}{T^2}$$

Enter hole size in mm	R	5
Enter pitch in mm	T	8

Open area based on the typed information = 35,4 %

The choice of hydroponic solution was based on a comparison of 3 established compositions:
UoG-CESRF (Masot et al., 2004)
USU (Utah State Univ. Crop Physiol. Lab. http://www.usu.edu/cpl/research_hydroponics.htm)
SKW (Research station for vegetable production, Sint-Katelijne Waver, Belgium, N. Ceustermans, personal communication)

Table 3: Comparison of nutrient solution recipes

	UoG (Masot *et al.*, 2004)	USU (dicot nutrient sol.)		SKW	
	mM	mM	mM	mM	mM
	vegetative	starter	vegetative	starter	vegetative
PO_4^{3-}	1.5	0.5	1.25	1.5	1.5
Ca^{2+}	3.62	1	2	3.9	3.8
NH_4^+	4	-	-	0.2	0.2
NO_3^-	11.75	3	7	9.8	10.3
K^+	5	1.6	4.45	5.5	6.5
SO_4^{2-}	2	0.5	1.5	0.6	0.6
Mg^{2+}	1	0.5	1.5	1.2	1.2
SiO_3^{2-}	-	0.1	0.1	-	-
	µM	µM	µM	µM	µM
Mn^{2+}	5	6	9	0*	5
Fe^{3+}	2.5	5	1.5	36.2	36.2
Zn^{2+}	3.5	6	4	5	5
B^{3+}	20	40	40	26	9.6
Na^+	8	0.2	0.2	-**	-
Cu^{2+}	0.8	4	4	1	1
Mo^{6+}	0.5	0.1	0.1	0.5	0.5

*Mn^{2+} is present in substrate used at start for SKW (peat/ soil mixture)
**Na^+ for SKW probably present in tap water but not quantified

Table 4: Nutrient composition of a commercial soluble fertilizer

Peter's	mM	Peter's	µM
P	0.75	Mn	5
Ca	3.1	Fe	20
N	7.2 (<1% NH_4^+)	Zn	1

K	2.7	B	24
S	1.8	Cu	1.3
Mg	0.6	Mo	0.5

Peter's 5-11-26 (Hydrozol; http://www.scottsprohort.com/) plus $CaNO_3$ and Fe-EDDHA was used to grow lettuce hydroponically under controlled conditions (Frantz et al. 2004).

Table 5: UG-HSB nutrient solution

UG-HSB	mM	UG-HSB	µM
P	1.5	Mn	5
Ca	3.6	Fe	5
N	10.7 (0% NH_4^+)	Zn	1
K	5.2	B	20
S	1.2	Cu	5
Mg	1.2	Mo	0.5
		Cl	10

Product	final conc	Product	final conc
	mM		microM
Ca(NO3)2.4H2O	3,6	Iron (Fe-EDDHMA) 6.0%	5
K(NO3)	3,5	MnSO4.H2O	5
KH2PO4	1,5	CuSO4.5H2O	5
MgSO4.7H2O	1,2	ZnSO4.7H2O	1
KOH	0,2	EDTA.2H2O.Na2	15
		Na2MoO4	0,5
		H3BO3	20
		KCl	10

1.4.2. pH and EC control

UoG:
EC = 1.9 dS m^{-1}
pH adjusted with 1 M $NaHCO_3$ till about 5.5
Total solution refreshed every 5 days.
USU:
pH adjusted daily with 1 M HNO_3 till 5.5 – 5.8
SKW:
pH adjusted online with 1 M HNO_3 till 5.8
EC = 1.5-2 dS m^{-1}

1.4.3. Iron availability

Fe^{3+} is supplied as $FeCl_3$ in the UoG recipe and as Fe-DTPA for USU and SKW.

1.4.4. Calcium availability

The commercial Peter's solution was chosen in the Frantz et al., 2004 research for its low NH_4^+ content, which helps to increase plant Ca^{2+} uptake. Reagent grade $CaNO_3$ was added because technical (fertilizer) grade has significant contamination with NH_4^+.
The UG-HSB solution was adapted from the SKW recipe with minor modifications and was made using reagent-grade chemicals.

1.4.5. Germination tests

Initial germination tests were carried out to determine if the seeds can be sown immediately on the growing medium. Germination efficiency was indistinguishable between filter paper moistened with double distilled water (produced onsite, conductivity less than 2microSiemens/cm) or nutrient solution. Moreover direct germination on small moistened (with nutrient solution) rock wool cubes proved successful, irrespective of vernalization at 4 ºC for 48 - 72 h in the dark. Germination took place at the same conditions as proposed for growing: continuous light, minimally 350 PAR, 25 ºC and 75% humidity. Seedlings to be grown in experiments will be selected on even germination time and even growth during the seedling stage to assure a homogenous crop (Korkmaz et al. 1999). EC and pH will be kept stable automatically (see above). The EC value of the nutrient solution will vary dependent on the growth stage: a lower EC value for the seedling stage and a higher one for the vegetative growth phase. The pH will be automatically maintained between 5.5 and 5.8.

2. Experimental plan

The two installed hydroponic circuits which can each hold 42 lettuce plants (2.25 m^2 growing area each). One circuit will be used for the stress treatment (*Pythium* or ACC) and the other for the control. In case ethylene gas would be used as stress condition instead of ACC addition to the nutrient solution, then the control and the treatment will need to be run consecutively and can therefore consist of 84 plants maximal.
The plants will be sown on rock wool plugs (1.5-1.5-4 cm, L-W-H) and germinated under growing conditions. A double amount of seeds (as compared to the amount of plants needed) will be used in order to allow selection of comparably developed seedlings. Germination will be stimulated and synchronised by a 2 day cold treatment (vernalisation at 6 degrees). After 7 days evenly germinated (within first two days) and evenly grown seedlings will be selected and placed in the gullies. When the plants are 14 days old the stress will be applied (see 1.2.1). ACC of the nutrient solution will be kept at 10 µM, based on the measured crop water usage info (sum of volumes of distilled water, concentrated feeding solution and acid supplied to the feeding tank of the hydroponic system), and assuming 100% conversion within the plant (root).

2.1. Tentative timing of destructive sampling

Destructive sampling will be carried out weekly on 5 individual plants per treatment.

Table 6: Experimental plan - stressed and control plants in same run

Day	Procedure	Sample size	#plant/treatment
1	sowing		100
7	Selection seedlings and start in gully		42
	Destructive sample	5	
14	Stress application		
	Destructive sample	5	37
21	Destructive sample	5	32
28	Destructive sample	5	27
35	Destructive sample	5	22
42	End harvest	22	0

Table 7: Experimental plan - stressed and control plants in consecutive run

Day	Procedure	Sample size	#plant/treatment
1	sowing		200
7	Selection seedlings and start in gully		84
	Destructive sample	10	
14	Stress application		
	Destructive sample	10	74
21	Destructive sample	10	64
28	Destructive sample	10	54
35	Destructive sample	10	44
42	End harvest	44	0

2.2. Parameters for destructive sampling

The following parameters will be determined for each destructive sampling:

-LAI (leaf area index) of individual plants
-Fresh weight of individual plants
Total, root and shoot weight
-Dry weight of individual plants
Total, root and shoot weight
-Microscopic observation of ethylene response (fungal intrusion)
-Q-PCR quantification of ethylene response based on the level of expression of ethylene-responsive genes (fungal growth and biomass based on the level of expression of fungal genes)

Nutrient composition of the tissues will be determined only at the end of at least one experiment (same sample as for weight determination). At the same time a sample from the nutrient solution will be analysed.

Table 8: Destructive sampling plan

Destructive Sampling method		**Destructive Sampling method**	
Fresh weight	root	**Microscopy**	Selective sampling
	shoot	**Q-PCR**	Selective sampling
Dry weight	root		
	shoot	**Nutrient content**	plant
Leaf area LAI	shoot		solution

2.3. On-line logged parameters

The in Table 9 listed parameters will be logged and thus be continuously available for experiment supervision.

Table 9: Parameters to be determined during an experimental run

	Frequency logging	**Online/ Manual**
Light quantity	10 min	Online
Temperature	10 min	Online
Humidity	10 min	Online
CO_2 in air	10 min	Online
O_2 in air	10 min	Online
Ethylene	10 min	Online
Calcium in solution	1 day	Manual
Oxygen in solution	1 day	Manual
pH	1 min	Online
EC	1 min	Online
Weight gully	10 min	Online

Condensate produced	1 min	Online
Nutrient stock solution used	1 min	Online
Water stock used	1min	Online
Acid stock used	1 min	Online
Video imaging	1 h	Online
Chlorophyll Fluorescence Imaging	1 h	Online
Thermal imaging	1 h	Online
Plant height	10 min	Online

Light quantity will be measured online for at least one position. Manual measurements at several points for a shelf will be determined at the beginning and end of a growing cycle.
This might be replaced by automated measurements at several positions and in function of time by placing the PAR sensors on the robot arm. This will generate a 3D map of light quantities throughout the growth space. However the cabling available for the setup described in TN 89.51 is not designed for repeated bending in the cable guides of the robotic system; this change has to be planned for a future upgrade of the system.
The positioning of the imaging system could be adapted to plant height evolution (growth) by adding a small plant height monitoring system to the robotic arm (e.g. laser range finder). Obviously the frequency of measurement woul equal that of imaging, which would anyhow suffice for the logging of plant growth.
Plant height monitoring was not implemented due to technical difficulties associated with precise aiming at plant surfaces of ultrasonic or optoelectric sensors, originally destined to be interfaced with the DeltaT logger (hence the mentioned 10min logging interval or uniformity with the other parameters).

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