ICP Vegetation
Ozone Sub-programme
2006

Experimental Protocol
for monitoring the incidences of
ozone injury on vegetation

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Working Group on Effects
of the
Convention on Long-range Transboundary Air Pollution

The ICP Vegetation is the International Cooperative Programme
on Effects of Air Pollution on Natural Vegetation and Crops

ICP Vegetation Experimental Protocol for monitoring the incidences of ozone injury on vegetation

2006

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1. Introduction

Reporting to the Convention on Long-range Transboundary Air Pollution, the ICP Vegetation is an International Cooperative Programme investigating the effects of air pollutants on vegetation in countries in the geographical region of the United Nations Economic Commission for Europe (UNECE), including Europe, USA and Canada. The Convention secretariat is with the UNECE. The main activities of the ICP Vegetation are as follows:

- Conducting coordinated experiments to determine the effects of ozone pollution on crops and (semi-)natural vegetation in Europe and North America.
- Developing models to quantify and interpret the influence of climatic conditions and environmental stresses on the responses of plants to ozone.
- Developing maps showing where vegetation is at risk from ozone pollution within the ECE region.
- Assessing the economic losses caused by the effects of ozone on crops.
- Collating and reviewing information on the effects of ozone on plant biodiversity.
- Considering the evidence for effects of nitrogen deposition on communities of (semi-) natural vegetation in Europe, including its modifying effect on the impacts of ozone.
- Collating and reviewing monitoring data on the atmospheric deposition of heavy metals, and subsequent accumulation by plants including a European survey of heavy metal concentrations in mosses.
- Studying the extent of nitrogen deposition in Europe by determining the nitrogen concentration in mosses at an extensive network of monitoring sites.

This Experimental Protocol provides the methodology to be adopted by participants in the ICP Vegetation for monitoring the frequency of ozone injury on white clover (*Trifolium repens* L cv Regal), brown knapweed (*Centaurea jacea*), commercially grown crops and naturally occurring (semi-)natural vegetation. The monitoring manual for sampling mosses and determining their heavy metal and nitrogen concentration is available as a separate document from icpvegetation.ceh.ac.uk.

This year we anticipate that fewer sites will monitor the frequency of ozone injury on *Trifolium repens*. Instead, participants are kindly requested to collate evidence from documents, surveys and internet sources in their native language, that ambient ozone has caused effects on crops and natural vegetation species from experiments and surveys from 1990 onwards. This work will be coordinated by Felicity Hayes, with assistance from Ben Gimeno (or colleague), Franc Batic, Jürg Fuhrer, Per Erik Karlsson and Håkan Pleijel. For further information please contact Felicity Hayes (fhay@ceh.ac.uk).

2. White clover

2.1 Experimental aims

- *To determine the geographical extent of the ozone problem within the UNECE region by identifying areas where the ozone concentration is sufficient to induce ozone-specific injury in a sensitive biotype of white clover*
- *To determine the frequency of ozone injury causing episodes at participating sites and to examine temporal trends*
- *To contribute data to a future revision of the short-term critical level of ozone for visible injury.*

2.2 Outline of the experiment

In this experiment, two batches of cuttings of ozone-sensitive white clover *(Trifolium repens cv* Regal, NC-S biotype*)* are distributed to participants from the Coordination Centre in the UK, at an interval of two weeks between the batches. Participants grow each batch of plants initially in a greenhouse for 28 days and then place them one plant per pot at an experimental site. After a further 28 days, the experiment starts by cutting the first batch of plants back to 7cm above the soil surface; the second batch of plants are cut back 14 days later. The presence of visible ozone injury (and other leaf damage e.g. by insects) is recorded at weekly intervals on both batches of plants using the method described. Each batch of plants should be cut back to 7cm every 28d until the end of the growing season (normally four or five such harvests). Leaf injury, biomass of the cuttings, climate and ozone data are sent to the Coordination Centre at the end of the growing season using standardised spreadsheets.

Figure 2.1 ICP Vegetation clover experiments

2.3 Experimental requirements

Greenhouse: For establishing clover cuttings prior to transplanting outdoors.

Experimental Plot: The site should be situated in an open field, at least 200m from main roads and 50m from buildings. The plot should be fenced to prevent birds, rabbits and small mammals from eating the clover and should be surrounded by grass or an artificial weed reducing material to prevent excessive dust formation, mud-splashes on the clover or overshadowing by weeds.

Replication: Use a minimum of 5 pots for each batch of clover plants.

Pots and wicks: Use 15 litre volume pots with a surface diameter of approximately 30cm. This pot size is needed because clover grows by 'trailing stems' (stolons) which root, therefore a lot of soil surface is required. Also, the 15 litre volume pots allow 5+ months of growth without pot binding. Fibreglass wick material is supplied by the Coordination Centre. Each pot should have access to a water reservoir as described below (Section 2.5)

Soil mixture and fertilizer: Participants to use whichever soil mixture/compost is appropriate for their area. Mixtures that work well in most areas are: peat, vermiculite, perlite and sand in the ratio 18:3:1:1 or local soil, sand and peat in the ratio 1:1:1. It is essential that participants use a slow release fertilizer such as 6 month slow release 'Miraclegrow' (formerly known as Osmocote, 13N:13P:13K) at a rate of 60 g per pot.

Plant Material: Cuttings of the NC-S clover will be supplied by the Coordination Centre.

Monitoring equipment: Ideally, participants should have access to pollution and climate data monitored at or close to the experimental site. Hourly mean data for ozone, temperature, humidity and solar radiation are the most useful.

2.4 Setting up and maintaining the experiment

2.4.1 Receiving and establishing the clover biotypes

Distribution of cuttings
Cuttings of the NC-S (ozone-sensitive) biotypes of white clover (*Trifolium repens*) will be supplied by either:

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Karine Vandermeiren
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Leuvensesteenweg 17
B-3080 Tervuren
Belgium

Tel: 32 276 922 33
Fax: 32 276 923 05
Email: *kavan@var.fgov.be*

You will be informed by the Coordination Centre of the approximate dates of arrival of the cuttings and will receive your cuttings in two batches of 20, fourteen days apart. If you are unable to plant the cuttings immediately, keep them refrigerated (but not frozen). Plant them as soon as possible in a greenhouse or outdoor growth chamber. The clover cuttings should be grown in 1 litre pots in the glasshouse for the first 28 days after planting as described below.

Planting procedure

The clover cuttings will be from virus-free stock, approximately 7 to 10 cm long, with 3 to 4 leaf nodes. Remove all but the youngest leaf by cutting (do not tear off).

Fill 1-litre pots with locally selected soil mixture. Mixtures that work well in most areas are peat, vermiculite, perlite and sand in the ratio 18:3:1:1 or local soil, sand and peat in the ratio 1:1:1. Before planting the cuttings, water the pots thoroughly to ensure the soil mix is fully wetted.

Use a pencil (or similar item) to make a hole in the medium. Rooting is better if this is at an almost horizontal angle, rather than vertical. Place the cutting in the hole so that about 6mm of the cutting (including apical meristem) remains above the surface. Firm the medium around the cutting and water-in. Label the pot to identify the biotype and delivery date.

Maintenance of the establishing cuttings

Rooting will begin immediately under reasonable conditions in the glasshouse/growth chamber. However, cuttings will suffer if the growth medium is too dry or too moist. They will need protection from dry, hot conditions (high sunlight levels), especially during the first week. If such conditions are anticipated, place the cuttings under a plant bench or in another protected area. High relative humidity is important. However, the cuttings probably won't need water for at least three days after starting. Be careful to avoid over-watering, particularly during the first 7-10 days as this can prevent the plants from becoming established. After this, over-watering may still increase the susceptibility of the biotypes to fungi.

Keep aphids and other insects off the plants to avoid the spread of viruses and diseases.

At 21 days after planting, fertilise each plant with 150ml of a water solution containing 1g/litre of Peters 5-11-26 (N-P-K) or close equivalent nutrient solution.

2.4.2 Transplanting

Twenty-eight days after planting the cuttings in the greenhouse they are ready to be transplanted to their final position in the experimental plot. Please note preparations for transplanting start *two days before* the plants are actually transplanted.

The minimum number of replicate plants to be placed at the experimental site per batch is five. However, it is advisable to grow more plants per batch in case some have to be discounted because of e.g. insect or slug damage.

For each batch, transplant the established clover plants into 15-litre pots (1 plant per pot) containing the chosen soil mix (see above), using the pot filling protocol described below. This will require 60g of Miraclegrow fertiliser (13:13:13 N-P-K, 6 month slow release formula) and four 60cm and one 120cm wicks per pot.

Pot-filling procedure

Safety Information: Strong gloves, a laboratory coat, eye protection and a dust mask should be worn at all times when handling the fibreglass wicks.

Two days before planting
Make five holes in the bottom of each pot, four near the edge and one in the centre as indicated in Figure 2.2. Pre-cut the fibreglass wick supplied into one 120cm length and four 60cm lengths per pot, and soak overnight in a bucket of water.

One day before planting
Fill the pots with the soil mix, inserting the wicks and applying the fertiliser using the following procedure:

1. Place the wicks inside the pot as indicated in Figure 2.2 with 20cm extending through each drainage hole and outside the bottom of the pot. 60cm wicks should be placed through the outer holes and the 120cm wick should be placed through the central hole.

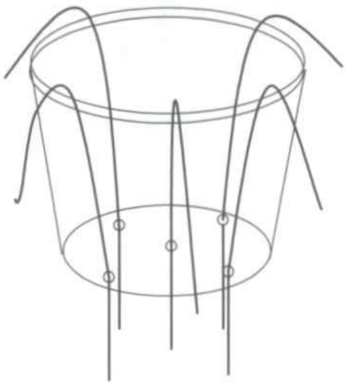

Figure 2.2 Placing the wicks in the pots

2. Place approximately 6-7cm depth of soil mix into the pot and pat down firmly. Add 12g of slow release fertilizer uniformly to the surface using a scoop calibrated to 12g. Lay one of the 60cm wicks clockwise in a partial circle approximately 2.5cm in from the pot perimeter (Figure 2.3).

Figure 2.3 Wind the first wick around in a clockwise direction

3. Repeat step 2 using the adjacent wick (clockwise) and including a further 12g of fertilizer as before.

4. Repeat step 3 twice.

5. Repeat step 3 for the remaining central wick, and fill with further soil mix until close to the top of the pot, taking care to ensure that the last wick cannot dry out by being too close to the surface.

6. Place the pots into the filled water reservoir and water the pots thoroughly from above, top up with soil mix as necessary to make the soil surface 4cm below the rim of the pot. The distance between the pot base and the water level should be approximately 3 cm; ensure that the pot base is not immersed in water. Drainage holes in the reservoir at this height would prevent this happening (Figure 2.4). The pots should be placed 0.5m apart within rows with 1m between rows.

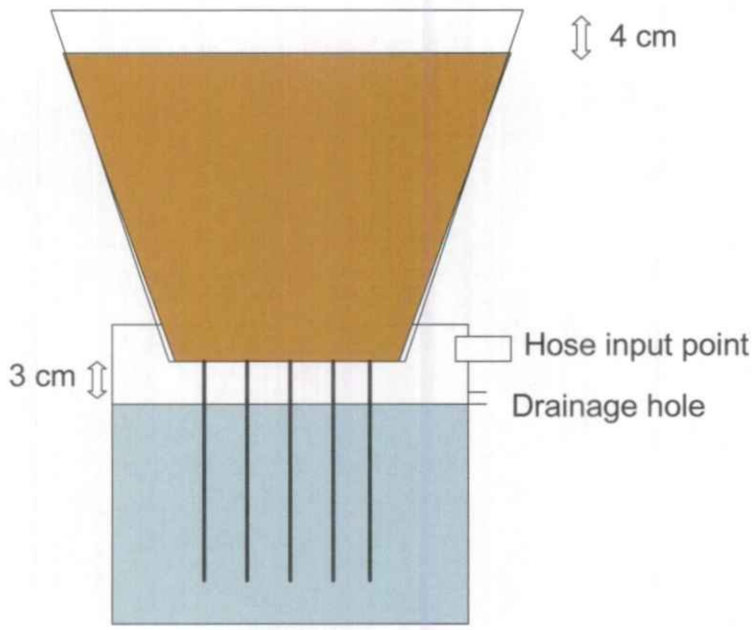

Figure 2.4 The pot and water reservoir system

7. In warm/hot climates, it is important to reduce over-heating by either sinking the pots and reservoirs in the ground, or by placing aluminised bubble-wrap (e.g. Reflectix) around the pots and reservoirs (if above ground).

Day of planting
Use a 1 litre pot (same as the clover is growing in) to make a 'template' hole in the middle of the 15 litre pot. Remove a 28 day old plant from its pot and place it into the 'template' hole. Firm the medium around the transplant and water thoroughly. Take care when transplanting the clover plants not to damage the shoots and roots. Standardise the surface of the soil mixture at 4cm below the top of pot for all pots (after saturation watering). The pots should be placed 0.5m apart within rows with 1m between rows.

2.4.3 Establishing the plants at the experimental site and first harvest

Day 1 of the experiment is the day that the clover plants are placed outdoors at the experimental plot and is thus the day of transplanting into the 15 litre pots. The plants are then given 28d to establish in the 15 litre pots before assessments are made. At the end of this period, the plants are cut back to 7cm above soil level as described in Section 2.5.3.

2.4.4 Care of clover plants

Watering: The clover plants are watered via the wicks that project into the water reservoirs and natural rainfall. The reservoirs should be checked daily, especially during the last week prior to cutting back to ensure the plants are watered sufficiently. Also ensure that the media is not holding too much water so that it is waterlogged.

Weeding: Remove weeds from the pots and around the pots as necessary.

Damaged plants: Remove any pots from the experimental plot if their clover plants become severely damaged by anything other than ozone pollution. Do not continue to harvest these.

Insect Pests
Careful observation for insect pests is required, for example, aphids sometimes hide below stolons coiled around the inside of the pot rim. A suggested insecticide is Avid (Manufactured by Syngenta) for control of aphids, thrips, leaf miners and red spider mites.

Viruses
Remove any affected plants from the site.

Fungal infections
If the plants are infected by a fungal pathogen, fungicides can be used but please ensure that the chosen chemical will not have an interaction with ozone. For example, it is believed that benomyl and triadimefon offer some protection against ozone, and thus these and chemically-related pesticides should not be used. Leaf Spot fungi can be a problem when there is a lot of rain during the growing season and Chlorothalonil (Bravo) is suggested for control. Systhane can be used to control mildew. Neither of these fungicides are thought to alter the response of the clover to ozone.

Herbivores
Birds, rabbits and deer feed on clover. A rabbit exclusion fence and bird netting is a requirement where these pests exist. For slug control use slug pellets (metaldehyde), or commercial biological control methods eg 'nemaslug' (nematodes).

Advice
Advice on any aspect of the experimental work can be sought from the Coordination Centre by emailing Felicity Hayes (fhay@ceh.ac.uk) or Phil Williams (pdwi@ceh.ac.uk). If requesting help with identifying symptoms of any type, please send photos with your email.

2.4.5 Environmental measurements

Ideally, the following information should be collected at each site using a computer-controlled monitoring system. Participants without monitoring facilities should provide any available information from a nearby monitoring site. Even if you are unable to provide any monitoring information for your site, please participate in the experiments because it may be possible to use information from other sites to aid in the interpretation of your results. Ideally, the following measurements should be made at 1m height, although data from other measurement heights is acceptable:

Physical climate

Air temperature (°C)
Relative humidity (%) or vapour pressure deficit (kPa)
Global radiation (Wm^{-2})
Rainfall: daily record of amount of rainfall (mm).
Wind speed ($m\ s^{-1}$) and direction (°)

Pollution Climate

It is recommended that continuous measurements of pollutant concentrations be made at the experimental site. Recommended methods are listed below. Passive sampling (e.g. diffusion tubes, lead dioxide surfaces) or records from nearby sites can be used as alternatives if continuous analyzers are not available.

Recommended methods:

Ozone	Gas-phase chemiluminescent, Gas-solid chemiluminescent, UV photometric
Sulphur dioxide	Flame photometric, UV absorption
Nitrogen oxides	Chemiluminescent

It is essential that gas analysers are regularly calibrated according to manufacturer's instructions. To ensure that the pollutant data is of adequate quality we will be requesting information on the method and frequency of calibration of analysers when we collect data from participants.

2.5 Assessments

2.5.1 Identifying visible ozone injury on white clover

The photographs presented in Figure 2.5 provide examples of ozone injury on white clover. These injury symptoms appear first on the upper leaf surface as fine pale yellow specks. Ozone injury is classed as present when 20% of the plants have one or more injured leaves. Please note that injury caused by red spider is similar in appearance but is accompanied by webbing and the presence of minute insects (c. 1mm diameter) on the lower surface of the leaf.

Figure 2.5 Examples of ozone injury on white clover

2.5.2 Weekly injury assessments

The following assessments should be made on the same day per week and prior to cutting back at the 28d harvests. If possible, daily observations of the plants should be made so that the date of first occurrence of ozone injury within each 28d period can be noted.

Assess each plant for ozone injury every week using the following scale:

- 0: no injury
- 1: very slight injury; occurrence of the first symptoms
- 2: slight injury, 1-5 % of the leaves with slight injury
- 3: moderate injury, 5-25 % of the leaves with injury
- 4: heavy injury, 25-50 % of the leaves injured
- 5: very heavy injury, 50-90 % of the leaves injured
- 6: total injury, 90 –100 % of leaves are injured

Grade each plant as either healthy (H) or abnormal, with the cause of the abnormality graded as 1 (slight), 2 (moderate), or 3 (severe) using the following key:

- S Stunted
- D Diseased
- I Insect damage
- Sl Slug damage
- A Animal (rabbits, deer, birds etc.)
- V Virus

If the clover plants are damaged by anything other than ozone pollution, please indicate on the recording form whether you think the data obtained at this harvest should be used.

2.5.3 28 day harvests

The cultivar of white clover used in this experiment grows rapidly. The plants need to be cut back every 28 days to ensure the development of new leaves for exposure to ambient ozone and injury assessment. By having two batches of plants established 14d apart, the presence of a near-full/full canopy is ensured throughout the experimental season.

Before the harvest, take photographs of the plants to give an indication of overall health and plant size.

Harvest procedure.
Use a guide stick with two lines; one line to indicate the soil surface and the second 7cm above the soil surface. Use long-blade garden shears to harvest the forage (leaves, flowers and stolons) at a height of 7cm above the soil surface. Also, harvest forage (runners) that have grown outside the pot perimeter. Remove any injured leaves which remain after cutting so that these cannot be mistakenly identified as having new injury at a subsequent observation (do not include these leaves in any biomass determination). Dip the shears into 10% Clorox solution between pot harvests to prevent virus spread from plant to plant (use manufacturers recommended safety precautions).

Put all the plant material from each pot into a labelled paper bag, dry to constant weight and record the weight. This is useful information for quality assurance checks on the data.

Repeat this harvest procedure on at least three dates at 28 day intervals after the first cut-back.

2.6 Optional additional measurements and experiments

2.6.1 Contributions to the development of a canopy flux-model for white clover

No further stomatal conductance data is required for clover as the modelling is now at an advanced stage. However, any measurements of leaf area index and or biomass increase during the 28d harvest periods would be invaluable. Please contact Felicity Hayes (fhay@ceh.ac.uk) for further details.

2.6.2 Biomass measurements for NC-S and NC-R biotypes of white clover

At sites with high ambient ozone, further data for biomass-response relationships can be collected by comparing the biomass of NC-S (ozone sensitive) with NC-R (ozone-resistant) biotypes of white clover. This can be achieved by growing NC-R clover alongside one batch of the NC-S clover used for the injury assessment experiment. The method for growing and assessing the plants is as described above. It is recommended that NC-S plants alternate with NC-R plants at the experimental site and that a minimum of 10 plants per biotype is grown.

Please contact Felicity Hayes (fhay@ceh.ac.uk) if you wish to participate in this experiment.

2.7 Data collection

We request that all data is returned to Felicity Hayes at the Coordination Centre by the end of November to ensure that it can be processed before the next Task Force Meeting, early in the following year.

Pollutant and climate data: should be sent by email to Felicity Hayes (fhay@ceh.ac.uk) as a spreadsheet preferably in Excel, Lotus, or ASCII text file format. The file should have a separate row for each hour of data i.e. the spreadsheet should have parameters such as ozone concentration and temperature along the top, and day and hour in separate columns down the left-hand side. Please fill in gaps in the hourly data according to the gap-filling rules provided by the Coordination Centre and clearly identify filled gaps with a change in font colour. This data should then be further processed to calculate daily means, daily minimums, daily maximums, 12 hour means, 7 hour means and AOT40s according to the processing rules provided by the Coordination Centre. Sample spreadsheets will be sent to participants. Preferably, please send data for the period from March or April though to October. A receipt will be sent for any data received.

Please note: due to time constraints it will not be possible to process files that are not in the above format and/or are sent as multiple files that need joining together and gap filling.

Plant data: should also be sent to Felicity Hayes by email on the spreadsheets provided at the start of the season. These require only the mean and standard deviation for each treatment and comments on the results to be included.

3. Surveys of naturally occurring vegetation for ozone injury

Survey natural vegetation in your area shortly after visible injury has developed on the clover or *Centaurea jacea* plants being used in the monitoring experiments. Suggested species to look at include:

Hypericum perforatum
Lotus corniculatus
Plantago lanceolata
Sanguisorba minor
Centaurea jacea
Cirsium arvense

Record injury as present or absent and **take photographs** of any injury observed. Record as many as possible of the following:

- Stage of development (e.g. flowering, seed set)
- Location
- Habitat, e.g. wetlands, meadow, commercial field
- Recent weather and pollutant conditions
- Soil type
- Altitude

Photographs of ozone injury on a range of species can be found at: http://www.ozone.wsl.ch/.

4. Monitoring the incidences of ozone injury on commercial crops

The aim is to determine the frequency of occurrence of injury-causing episodes on crops with special emphasis on those crops for which leaf injury would reduce their economic value. Surveys of commercial fields can be made and/or experimental crops can be grown at the experimental site. The wide range of crops for which ozone injury has been detected in the field are shown in Table 4.1.

Table 4.1 Commercial agricultural and horticultural crops injured by ambient ozone episodes.

Agricultural crops		Horticultural crops	
Bean	*Phaseolus vulgaris*	Courgette	*Cucurbita pepo*
Clover	*Trifolium repens*	Chicory	*Chicorium endiva*
Corn	*Zea mays*	Lettuce	*Lactuca sativa*
Grape-vine	*Vitis vinifera*	Muskmelon	*Cucumis melo*
Peanut	*Arachis hypogea*	Onion	*Allium cepa*
Potato	*Solanum tuberosum*	Parsley	*Petroselinum sativum*
Soybean	*Glycine maxima*	Peach	*Prunus persica*
Tobacco	*Nicotiana tabacum*	Pepper	*Capiscum anuum*
Wheat	*Triticum aestivum*	Radish	*Raphanus sativus*
	Triticum durum	Red beetroot	*Beta vulgaris*
		Spinach	*Spinacea oleracea*
		Tomato	*Lycopersicon esculentum*
		Watermelon	*Citrullus lanatus*

Participants are encouraged to monitor ozone-induced injury on those species that are commercially grown in their area following incidences of ozone injury on the experimental clover and/or *Centaurea jacea*. If possible, please record the crop and cultivar, date, extent of injury and recent weather conditions. Take photos of any injury.

In addition, participants could grow a number of plants from seeds obtained locally and expose them to ambient air when they have reached sufficient size using the same protocol as used for clover (Section 2). Record the extent of ozone-induced injury on these species weekly using the assessment procedure described for clover. Shortly after injury symptoms have developed on the trial plants, please contact local commercial growers and find out whether any damage has occurred in their fields/glasshouses.

5. Monitoring the incidence of ozone injury on clones of brown knapweed (*Centaurea jacea*)

Contact person:

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Tel: +41 1 377 7509

Agroscope ART
Air Pollution/Climate Group
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Switzerland

Additional contact:

Prof. Dr. Jürg Fuhrer juerg.fuhrer@fal.admin.ch
Tel: +41 1 377 7505

5.1. General information

5.1.1 Experimental aims

- *To test the use of sensitive and resistant clones of Centaurea jacea for biomonitoring in the UNECE area.*
- *To determine the geographical extent of the ozone problem within the UNECE area by identifying areas where the ozone concentration is sufficient to induce ozone-specific injury on brown knapweed (Centaurea jacea), a species of (semi-)natural vegetation.*
- *To develop a short-term critical level for the development of visible injury on a species of (semi-)natural vegetation.*

5.1.2 Outline of the experiment

Centaurea jacea is a species of (semi-)natural vegetation that is widespread in Europe. A biomonitoring method has been developed in which the presence of ozone injury on the rosette and stem leaves of pot-grown plants is assessed at weekly intervals. To reduce genetic variation among individuals, a sensitive and resistant clone have been developed for intended use within the program and the 2006 season will act a widespread field trial for the two clones. Sensitive and resistant clones in the form of plantlets are provided by Agroscope ACW Changins, Switzerland and exposed to ambient ozone when at least 8-10 rosette leaves have developed. The exposure period lasts approximately three months.

Figure 5.1 A mature *Centaurea jacea* plant at the end of the exposure period. The measuring stick is one metre. Photo by Linda Schenk, Östad Sweden, 2003

5.1.3 Experimental requirements

Experimental plot: The site should be situated in an open field, at least 200m from main roads and 50m from buildings. The plot should be fenced to prevent birds, rabbits and small mammals from eating the brown knapweed.

Replication: At least 10 sensitive (S) and 10 resistant (R) *Centuarea jacea* clones should be exposed at each experimental site.

Pots and wicks: As for clover, use 15-litre pots with a surface diameter of approximately 30cm. This pot size is required because *Centaurea jacea* is a hemicryptophyte producing a large root volume for hibernation. Use the same fiberglass wicks (provided by the Coordination Centre) and water reservoirs as for the clover plants.

Soil mixture: Use a 1:1:1 mixture of sand, peat, and local garden soil.

Fertilizer: To ensure that the plants are not nutrient limited, we suggest adding some Nitrogen and basic nutrients to the pots. Over the course of the 3 month exposure, a total of 30 kg Nitrogen ha^{-1} yr^{-1} should be applied to each pot. N should be added every two weeks as ammonium-nitrate, NO_3NH_4, in 0.5 liters of water (0.5 liters was chosen to avoid loss of solution due to drainage through the bottom of the pot), starting as soon as the plants are exposure outdoors at the experimental site (5 kg N ha^{-1} yr^{-1} x 6 applications). Application rates should be calculated according to the surface area of the pots being used. To ensure that other basic nutrients are not limiting, Hoagland's No. 2 basal salt mixture (Sigma-Aldrich product no. H2395) should also be added to the same 0.5 litre solution of ammonium-nitrate fertilizer. (See example in **section 5.2.3** below for details).

Clones: The clones were produced via micropropagation from *Centaurea jacea* found in extensive meadows in the Canton of Geneva in Western Switzerland, and will be supplied by Agroscope ACW Changins, Switzerland.

Monitoring equipment: Ideally, participants should have access to pollution and climate data logged at or close to the experimental site. Hourly mean data for ozone, temperature, humidity and solar radiation are the most useful.

5.2. Setting up and maintaining the experiment

This consists of two phases:

1. Acclimatisation of the plantlets (*Centaurea jacea*)
2. Cultivation the plants during the experimental phase

General schedule:

Acclimatisation after reception; 6 – 8 weeks	Repotting and adapting to exterior; 2 –3 weeks	Experimental phase ICP Vegetation Experimental Protocol

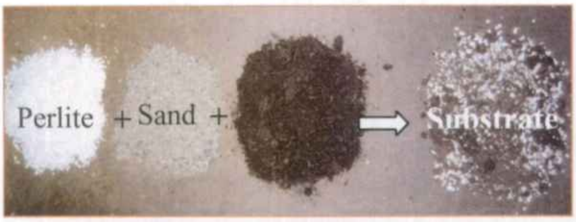
5.2.1 Early cultivation of *Centaurea jacea*

You will be informed of the approximate arrival date of the cloned brown knapweed (*Centaurea jacea*) plantlets. **Please note:** the plantlets are delicate and should be attended to immediately upon arrival.

Details for transferring the *in vitro* plantlets to small pots and then outdoor conditions are outlined below (prepared by Agroscope ACW Changins, Switzerland).

At reception

At reception the plantlets could be washed and be refreshed if necessary before the potting.



Potting

The potting substrate should have a good drainage and be free from weed seeds.

A possible substrate recipe :
¼ coarse sand, ¼ peat, ¼ garden soil, ¼ Perlite®

Cultivation pots : 6 – 8 cm plastic or clay pots

Put the plants with the roots vertically downwards in the pot and the collar and leaves above the substrate's surface.

Mist

The acclimatisation of the plants is relatively easy and can be done in room conditions provided enough daylight is provided. The best would be to place the plants close or next to a window at room temperature (~20^{0}C).

After watering the plants will be placed in a transparent plastic bag and set by the window. The plastic bag should itself be closed as to not let any important gas exchanges take place with the room environment for the next 4 days. Keep the substrate sufficiently humid.

Hardening

After 4 days, one can open one end of the plastic bag and slowly accommodate the plants to the room conditions. On the 7th day after planting, the plastic bags may be removed altogether.

Culture

After hardening, and once new leaf growth is observed, the plants will be watered less. Over the following weeks the plant care should progressively become adapted to outdoor climatic conditions. The species needs little or no fertilising.

Planting out

Once the plants have attained their appropriate size for outdoor planting they should be adapted slowly to direct sunlight. This adaptation period can take some 2 to 3 weeks. It remains important to keep the plants sufficiently watered during all of this period.

5.2.2 Transplanting

At the 8-10 true leaf stage, transplant the *Centaurea jacea* clones into 15 litre pots using the method described below (as described for clover, section 2.4.2, using wicks and a water reservoir). This will require four 60cm and one 120cm wicks per pot. The soil mix to be used is sand, peat, and local garden soil in a 1:1:1 mixture and unlike for clover, **do not** add any slow release fertilizer to the pots. Please note preparations for transplanting start *two days before* the plants are actually transplanted.

Pot-filling procedure

Safety Information: Strong gloves, a laboratory coat, eye protection and a dust mask should be worn at all times when handling the fiberglass wicks.

Two days before planting

Make five holes in the bottom of each pot, four near the edge and one in the centre as indicated in Figure 5.2. Pre-cut the fiberglass wick supplied into one 120cm length and four 60cm lengths per pot, and soak overnight in a bucket of water.

One day before planting

Fill the pots with the soil mix, inserting the wicks using the following procedure:

1. Place the wicks inside the pot as indicated in Figure 2 with 20cm extending through each drainage hole and outside the bottom of the pot. 60cm wicks should be placed through the outer holes and the 120cm wick should be placed through the central hole.

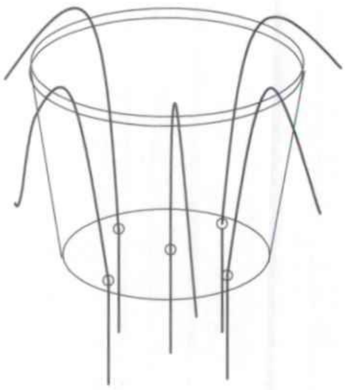


Figure 5.2. Placing the wicks in the pots

2. Place approximately 6-7cm depth of soil mix into the pot and pat down firmly. Lay one of the 60cm wicks clockwise in a partial circle approximately 2.5cm in from the pot perimeter (Figure 5.3).

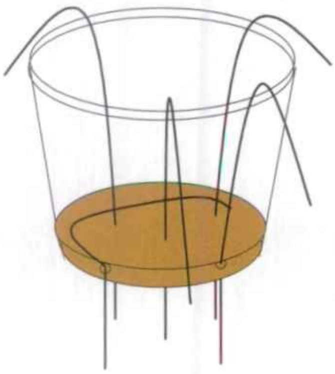


Figure 5.3. Wind the first wick around in a clockwise direction

3. Repeat step 2 using the adjacent wick (clockwise).

4. Repeat step 3 twice.

5. Repeat step 3 for the remaining central wick, and fill with further soil mix until close to the top of the pot, taking care to ensure that the last wick cannot dry out by being too close to the surface.

6. Place the pots into the filled water reservoir and water the pots thoroughly from above, top up with soil mix as necessary to make the soil surface 4cm below the rim of the pot. The distance between the pot base and the water level should be approximately 3 cm; ensure that the pot base is not immersed in water. Drainage holes in the reservoir at this height would prevent this happening (Figure 5.4). The pots should be placed 0.5m apart within rows with 1m between rows.

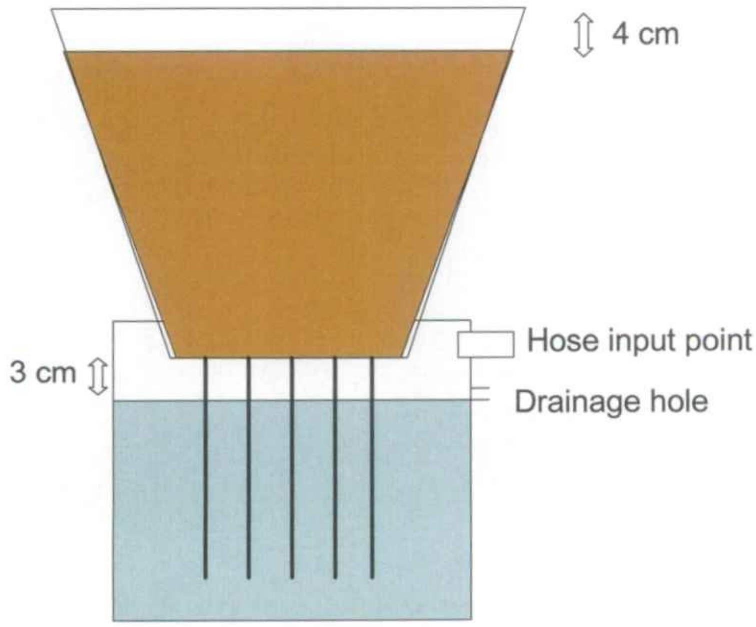


Figure 5.4. The pot and water reservoir system

7. In warm/hot climates, it is important to reduce over-heating by either sinking the pots and reservoirs in the ground, or by placing aluminized bubble-wrap (e.g. Reflectix) around the pots and reservoirs (if above ground).

Day of planting

Use a 6-8 cm pot (same as the plantlets are growing in) to make a 'template' hole in the middle of the 15 litre pot. Remove the Centaurea plant from its pot and place it into the 'template' hole. Firm the medium around the transplant and water thoroughly. **Take care NOT to damage or alter the root systems in their current pot-like form.** Standardise the surface of the soil mixture at 4cm below the top of pot for all pots (after saturation watering). After the re-potting, the plants should be adapted over the next 2 to 3 weeks to direct sunlight. This can be done by placing the plants in full shade and, progressively, reduce the shade until full sun exposure. This must also be done with care as plants like people coming out of the long winter months are very light sensitive and may suffer severe burns. This adaptation period should take some 2 to 3 weeks. It remains important to keep the plants sufficiently watered during all of this period!

The pots should be placed 0.5m apart within rows with 1m between rows.

5.2.3 Care of the *Centuarea jacea* plants

The *Centaurea jacea* plants should be maintained throughout the experiment as described for clover in Section 2.4.4. of this protocol.

Fertilization details:

Over the course of the 3 month exposure, a total equivalent of 30 kg Nitrogen ha^{-1} yr^{-1} should be applied to each pot. This would mean 3 g N /m^2/yr. A 30 cm diameter pot has an area of 0.071 m^2 and thus a total of 0.21 g N per pot should be added. When divided by 6 applications (every 2 weeks for 3 months), this would amount to 0.036 g N per pot for each application. In terms of ammonium-nitrate, NO_3NH_4, this would be **0.103 g NO_3NH_4 /pot/application**. It may be easier to prepare 10 liters of solution which can then be used for all 20 pots (0.5 l per pot), which would require 2.06 g of NO_3NH_4 per 10 liters of water. **Please note:** *These calculations are based on a pot with a 30 cm diameter. You may need to recalculate if your pot size is different.*

To ensure that other basic nutrients are not limiting, Hoagland's No. 2 basal salt mixture (Sigma-Aldrich product no. H2395) should also be added to the same 0.5 liter solution as the ammonium-nitrate fertilizer. The **manufacturer** recommended application is 1.6 g of Hoagland's powder per 1 liter of water. Since we suggest adding only 0.5 l of water, this would be a total of 0.8 g per 0.5 l of water. When divided by 6 applications (every 2 weeks for 3 months), this would amount to 0.13 g of powder per 0.5 l per pot for each application. When prepared in the same 10 liters as the NO_3NH_4 solution, you would need 2.6 g of Hoagland's powder. The product mentioned above is available in a container with enough of powder to prepare 10 liters of solution (indicated as 10 l) when applied at 1.6 g/l water. This amount should be enough for 20 plants over the course of the 3 month experiment.

(See: http://www.sigmaaldrich.com/catalog/search/SpecificationSheetPage/SIGMA/H2395)

5.3. Injury assessments and morphology

5.3.1 Identifying ozone injury on *Centaurea jacea*

Ozone injury is less clearly defined on *Centaurea jacea* than on white clover. Typical ozone stipple injury occurs (Figures 5.5-7) as well as a less specific reddening injury (Figure 5.8) that may be due to an ozone and climate stress interaction. The presence of both types of injury is to be assessed separately at weekly intervals.

Figure 5.5 Typical ozone-induced visible injury on the rosette of a sensitive clone of *Centaurea jacea* in comparison to a younger uninjured leaf.

Figure 5.6 Early stages of ozone-induced bronze stippling on a rosette leaf of a sensitive clone of *Centaurea jacea*.

Figure 5.7 Typical ozone-induced visible injury on the rosette of a sensitive clone of *Centaurea jacea*.

Figure 5.8 Reddening injury on *Centaurea jacea.* Note that both the lower and upper side of the reddening part can be seen; the discolouring goes all the way through the leaf.
Photo by Linda Schenk, Östad Sweden, 2003.

Figure 5.9 Comparison of sensitive (S) and resistant (R) clones of *Centaurea jacea* grown in the control and ozone fumigated climate chambers. S clones grown in the ozone fumigated chamber were the only plants to show ozone-induced bronzing (upper left panel).

5.3.2 Weekly assessments

This species first produces a rosette of leaves and then grows several flower stems. Weekly assessments of the amount of each type of ozone injury (stipple and reddening) should be made for both rosette and stem leaves.

<u>**Assessment of injury on rosette leaves:**</u>

Record leaf injury weekly by counting the total number of rosette leaves and the number of injured rosette leaves. The number of leaves showing typical ozone-induced injury, i.e. stippling, should be recorded separately from less distinct reddening. Leaves less than 5 cm in length should not be counted. To facilitate this, we suggest constructing a 10 cm diameter ring (cardboard, plastic, etc.) which can be placed over the center of the rosette and only those leaves extending beyond the ring are counted and assessed for ozone injury (Figure 10). The assessment of the rosette leaves for each plant should be conducted from the start of the exposure outdoors until the first stem leaf is marked and assessed. The assessment of the rosette leaves is then optional for the rest of the experimental period.

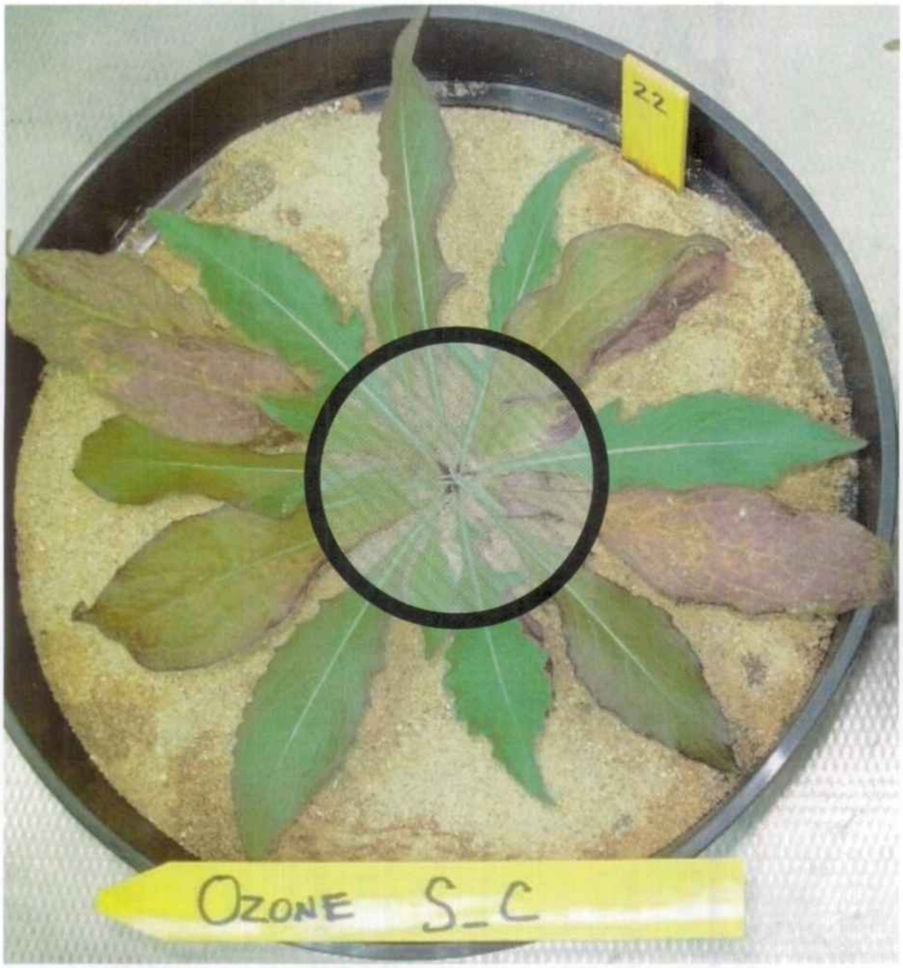


Figure 5.10 An example of a 10 cm diameter ring (cardboard, plastic, metal, etc.) which can be placed over the center of the rosette and only those leaves extending beyond the ring are counted and assessed for ozone injury.

Detailed assessment of injury on stem leaves:

The following procedure allows for a detailed assessment of the progression of stipple-injury on the individual stem leaves of *Centaurea jacea*.

1. As soon as the first shoot grows, mark it with a small tag (No. 1) (Figure 11).
2. Count and mark every second leaf from **bottom to top** (No.1, 3, 5, 7…).
3. Repeat this procedure with shoot No. 3 and shoot No. 5 if these develop.
4. Observe first occurrence and progression of visible injury on every marked leaf using the following assessment key and provided assessment form as shown in Table 1:

0: no injury
1: very slight injury; occurrence of the first symptoms
2: slight injury, 1-5 % of the leaf is injured
3: moderate injury, 5-25 % of the leaf is injured
4: heavy injury, 25-50 % of the leaf is injured
5: very heavy injury, 50-90 % of the leaf is injured
6: total injury, 90 –100 % of leaf is injured

Figure 5.11 Example of counting and marking the shoots of *Centaurea jacea* for the assessment of visible ozone injury.

Table 1. Example of a form for the detailed assessment of visible injury on *Centaurea jacea* per plant (an assessment form template in an Excel spreadsheet will be provided).

DATE:														
Plant No.	Status	Flower	Seed	Rosette			Leaf No.	Stem 1		Stem 3		Stem 5		
		y/n	y/n	Total	Stipple	Red		Stipple	Red	Stipple	Red	Stipple	Red	
							1							
							3							
							5							
							7							
							9							

If the *Centaurea jacea* plants are damaged by anything other than ozone pollution, please indicate on the assessment form whether you think the data obtained at this assessment should be used. The following codes should be used to grade the **status** each plant as either healthy (H) or abnormal, with the cause of the abnormality graded as 1 (slight), 2 (moderate), or 3 (severe) using the following key:

S Stunted
D Diseased
I Insect damage
Sl Slug damage
A Animal (rabbits, deer, birds etc.)
V Virus

In addition, please record whether or not the plant is flowering and/or has produced seeds.

5.4 Data collection

Plant data for the Centaurea experiment ONLY should be sent to Kris Novak (kris.novak@fal.admin.ch) using the datasheets provided at the start of the experiment.

Appendix 1. Additional Information about *Centaurea jacea* from Changins

Growth substrate

Before sending to participants this species has been grown in Agroscope-ACW Changins in the substrate mentioned below. This substrate is designed to give the plants a similar situation as they would find in the nature all whilst procuring sufficient drainage.

Substrate recipe used in Agroscope-ACW Changins:

¼ coarse sand	drainage
¼ peat	acidity and organic matter
¼ garden soil	nutrition
¼ Perlite®	drainage
Growth pots 6 – 8cm	

Outdoor cultivation details (general information)

Habitat

Centaurea jacea grows naturally in open areas such as pastures, slopes, sides of paths or well light woods. It may be seen in hedgerows, uncultivated land and waste ground. It is found up to an altitude of 2000 meters. The species has a Eurasian distribution.

In natural conditions *Centaurea jacea* prefers light (sandy), medium (loamy) and heavy (clay) soils, it requires well-drained soil and can grow in nutritionally poor soil. The plant prefers acid, neutral and basic (alkaline) soils and can grow in very alkaline soil. It cannot grow in the shade. It requires dry or moist soil and can tolerate drought.[1]

The species succeeds in ordinary garden soil; prefers a well-drained fertile soil and a sunny position; tolerates dry, low fertility and alkaline soils. This species is hardy to at least -15°C. [2]

For further information, please, don't hesitate to contact:

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www.racchangins.ch

[1] http://www.ibiblio.org/pfaf/cgi-bin/arr_html?Centaurea+jacea

[2] http://www.ibiblio.org/pfaf/cgi-bin/arr_html?Centaurea+jacea

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