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MEETINGS OF THE ROYAL BOTANICAL SOCIETY OF THE NETHERLANDS

MEETING OF THE SECTION FOR PHYTOPATHOLOGY ON 24 JANUARY 1985

H. HUTTINGA and W.H.M. MOSCH (*Instituut voor Plantenziektenkundig Onderzoek, postbus 9060, 6700 AA Wageningen*)

The use of molecular-hybridization techniques for the detection of plant viruses and viroids

In cooperation with the Department of Molecular Biology of the Agricultural University Wageningen, cDNA-probes were produced for potato spindle tuber viroid (PSTV) and potato virus Y^N (PVY^N) for use in molecular-hybridization tests. The probe for PSTV contains a full-length copy of the viroid-RNA: the probe for PVY^N contains a copy with a length of about 2500 base pairs, which is 25% of the total lenght of the PVY^N-RNA. The probes were labelled with ^{32}P by "nick-translation". Nitrocellulose filters were used as solid support. 3 µl samples were spotted onto dry filters that were pretreated with 20 × Standard Saline Citrate.

The molecular-hybridization test is a quick method – results can be obtained within 4-5 days – and only 3 µl of sample is needed. Moreover the method is cheap in comparison to the tomato-PAGE method.

The detection limit for PSTV using the cDNA-probe is 125-250 pg. This is sufficient to detect PSTV in crude sap of tomato leaves and in leaves of secundarily infected potato plants. For young primary infections in potato, concentration of the viroid from a larger sample will be necessary to obtain the desired sensitivity.

The detection limit for PVY^N using the molecular-hybridization test is 30 pg. This allows an easy detection of PVY^N in crude sap of potato leaves. Dilution experiments proved that even one diseased leaf in a bulk sample of 300 leaves can be detected. PVY^N can also be detected in dormant tubers just after lifting.

J.G.TH. DIBBITS and B.J.M. VERDUIN (*Vakgroep Virologie, Landbouwhogeschool, Binnenhaven 11, 6709 PD Wageningen*)

Detection of potato spindle tuber viroid with cDNA using non-radioactive labelling

Potato spindle tuber viroid (PSTV) complementary DNA in a pBR322-derived plasmid (VAN WEZENBEEK et al. 1982, *Nucleic Acids Research* **10**: 7947-7957) was modified with biotin by nick-translation in the presence of biotin-dUTP. Subsequently this biotin-modified DNA was detected with avidin which was conjugated to several non-radioactive labels: fluorescein isothiocyanate (FITC), horse radish peroxidase (HRP) and alkaline phosphatase (AP).

Serial dilutions of modified plasmid were dot-blotted on nitrocellulose paper and with all three labels down to 250 pg of plasmid could be detected.

Dot-blotting unmodified plasmid and introducing a hybridization step with modified plasmid increased the detection limits for all three labels to 500 pg of unmodified plasmid.

When serial dilutions of purified PSTV were dot-blotted on paper, hybridized with modified cDNA and visualized, the lower detection limit for all three labels was 30 ng of viroid RNA.

Increasing the percentage of substitution of thymidine residues with biotin-d-uridine and cross-linking of the alkaline phosphatase will offer possibilities for a 100-fold decrease of the detection limits.

A second method involved chemical modification of cDNA with 2,4-dinitrobenzaldehyde. This

aldehyde binds covalently to the nucleotides and the resulting modified DNA is recognized by antiserum against dinitrophenol (DNP). A second antiserum conjugated to alkaline phospatase was used to visualize the modified cDNA-primary antiserum complex. Less than 100 pg of modified cDNA could be detected with this method. Using this modified cDNA as a hybridization probe, 100 pg of unmodified homologous plasmid and 15 ng of purified PSTV were detectable.

H.J.M. LINTHORST[1], C. CUPERUS[2], C.J. ASJES[3] and J.F. BOL[1]
([1]*Biochemisch Laboratorium der Rijksuniversiteit Leiden,Postbus 9505, 2300 RA Leiden;* [2]*I.P.O., Postbus 42, 6700 AA Wageningen;* [3]*L.B.O., Vennestraat 22, 2161 LE Lisse*)

Homology between the genomes of tobacco rattle viruses from potato and tulip

Different TRV strains isolated in The Netherlands from potato (TRV strain PSG) and tulip (TRV strain TCM) were purified from infected tobacco, the RNA was extracted and polyadenylated and cDNA was synthesized with oligo dT10 as a primer in the reverse transcriptase reaction. After second strand synthesis the double stranded DNA was tailed with deoxycytosine and annealed in oligodeoxyguanosine tailed, linearized plasmids pBR 322 and pUC 9, after which these materials were used to transform *E. coli.*

Several clones (*fig. 1*) corresponding to RNA 2 of strains PSG and TCM were characterized by Northern blot hybridization.

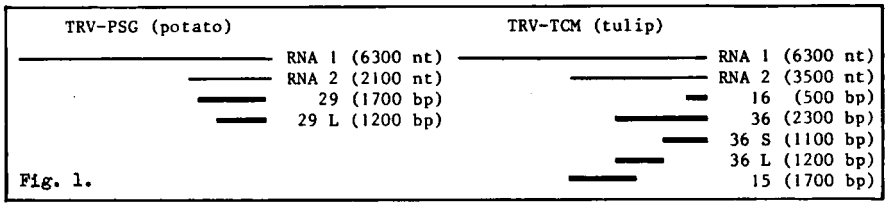

Fig. 1.

The PSG clone 29 L hybridized efficiently to RNA 2 of another local TRV isolated from potato (TRV-PNP) and the PRN-strain of TRV but only very weakly to RNA 2 of the tulip strain (TRV-TCM) and RNA 1 of all isolates tested. The TCM-clones 16 and 36 S hybridized efficiently to both RNA 1 and RNA 2 of the tulip strain (TCM) and all potato strains (PSG, PNP, PRN). The TCM-clone 36 L hybridized efficiently to TCM-RNA 2, weakly to TCM-RNA 1, but not to RNA of any of the potato strains. The TCM clone 15 hybridized exclusively to TCM-RNA 2.

From these results we conclude that RNAs 1 and 2 of the tulip strain TCM have a 3'-terminal homologous sequence of more than 1000 nucleotides, whereas the 3'-terminal homologous region in RNAs 1 and 2 of the potato strain is probably less than 500 nucleotides. Sequence analysis confirmed that the 3'-terminal nucleotide sequence of the RNAs of the PSG and TCM strain are almost identical.

With the available clones as probe we could detect picogram amounts of TRV in infected tobacco, potato and tulip tissues by dot blot analyses. By appropriate selection of the clones we can detect either one specific isolate of TRV or a whole spectrum op different TRV strains.

H. VAN PELT-HEERSCHAP[1], H. VERBEEK[1], J. W. SLOT[2] and L. VAN VLOTEN-DOTING[1] ([1]*Biochemish Laboratorium, Wassenaarseweg 64, 2333 AL Leiden;* [2]*Centrum voor Electronenmicroscopie, Padualaan 8, 3584 CH Utrecht*)

The constituents of the alfalfa mosaic virus replication complex.

The tripartite genome of alfalfa mosaic virus (AlMV, a ss RNA$^+$ plant virus) is only infectious when each of the genome segments is complexed with coat protein (SMIT et al. 1981). The coat protein binds specifically to the 3'-terminal homologous region of the RNAs (ZUIDEMA et al. 1984) and this binding is probably a prerequisite for RNA replication (HOUWING & JASPERS 1978).

As a first approach for the cellular localization of the viral replication complex we studied localiza-

tion of coat protein and minus stranded viral RNA. The coat protein of AlMV was localized in ultrathin frozen sections of *in vivo* infected tobacco protoplasts by immunochemical electronmicroscopy using colloidal gold as a cytochemical marker. Electronmicroscopical observation demonstrated gold particles (linked to Protein A bind to anti-coatprotein antibody) in the cytoplasma and the nucleus, especially around the nucleolus. With standard electronmicroscopy techniques AlMV particles have previously been visualized in the cytoplasma, but never in the nucleus. (HULL et al. 1969). From studies with ts mutants (SARACHU et al. 1983) it is known that the products encoded by the two largest genome segments are involved in RNA replication. For the cellular localization of these proteins antibodies are being produced against synthetic peptides (27 and 28 amino acids long) corresponding to hydrophylic regions of the proteins.

The localization of minus stranded viral RNA was investigated by Nothern blots of RNA, extracted from infected tobacco leave homogenates, purified nuclei and postmitochondrial fractions. End labelled fragments of genomic RNAs were used as a probe. No minus stranded RNA could be detected in purified nuclei. Indicating that the replication of AlMV RNAs does not take place in the nucleus.

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H. VELVIS
(Instituut voor Bodemvruchtbaarheid, Postbus 30003, 9750 RA Haren (Gn))

Survival of sclerotia of Rhizoctonia solani in soil

Sclerotia of *R. solani* were placed about 1 cm deep into two sands, two sandy loams and two clay loams, in petri-dishes, and kept at 10 or 20°C. At 10°C all sclerotia were still alive after 75 days. A considerable decrease in vitality, however, was observed at 20°C in the sands and in one sandy loam (from Kloosterburen), containing high densities of the mycoparasite *Verticillium biguttatum*. These soils had a pH-KCl of 5 or lower. Many sclerotia were also killed in one clay loam, apparently as a result of attack by parasitic nematodes. *V. biguttatum* appeared to be the most important cause of reduction in vitality. Accumulation of antagonistic bacteria and streptomycetes was not observed in any of the soils. In sand from Haren, *Gliocladium nigrovirens* presumably played a role, besides *V. biguttatum*.

Soil temperature greatly affects vitality; the minimum lies between 10 and 13°C; at higher temperatures activity increases rapidly. The rate of colonization and killing of sclerotia was high in the Haren sand at 20°C: within 2 weeks almost 90% of the sclerotia was parasitized by *V. biguttatum*, while 50% was killed. A small number of sclerotia, however, escaped colonization and survived for at least half a year. It was concluded that first-stage colonization by *V. biguttatum* governs survival of sclerotia.

G. JAGER *(Instituut voor Bodemvruchtbaarheid, Postbus 30003, 9750 RA Haren (Gn)*

Biological control of Rhizoctonia solani in seed potatoes: prospects and restrictions

The biological control of *Rhizoctonia solani* in potatoes by the mycoparasite *Verticillium biguttatum* can take place: 1° during storage of tubers contaminated with sclerotia of *R. solani* and 2° in the field during the growing season.

Destruction of sclerotia of *R. solani* by spraying a spore suspension of *V. biguttatum* on tubers when stored is successful during storage provided: 1° the tubers are free from adhering soil, 2°

the temperature during storage is at least 15 °C and 3° the relative humidity of the air between the tubers is 100% or close to it. The last demand is usually fulfilled.

The biological control in the field during the growing period of 3–4 months gives problems in soils with a high inoculum density of *R. solani* or in soils where *R. solani* has good possibilities for growth. (The sclerotium index of the harvest of disinfected tubers is used as a measure of the possibilities *R. solani* had during the growing season.) Usually in acid sands the reduction of the sclerotium index by *V. biguttatum* is too small, if it occurs at all, to be of interest. In neutral marine loam soils – where the majority of seed potatoes is grown – the biological control proved often successful. But also in these soils high population densities of *R. solani* reduce the effectiveness of biological control. Integrated control through a soil treatment with *R. solani* inhibitors as pencycuron (moncerene) or tolclofos-methyl at lower dosages than advised plus inoculation of the seed tuber with a spore suspension of *V. biguttatum* seems to be effective in reducing the sclerotium index of the harvest.

The relatively high minimum temperature required for growth of *V. biguttatum* (12 °C) is a drawback as was apparent in the cold spring and early summer of 1984; no activity was found and the presence of the organism on stolons of inoculated tubers decreased in the course of the growing season.

P. H. J. F. VAN DEN BOOGERT (*Instituut voor Bodemvruchtbaarheid, Postbus 30003, Haren (Gn)*

Quantitative assessment of Verticillium biguttatum in soil

This investigation was supported by the Netherlands Foundation for Technical Research (STW), future Technical Science Branch/Division of the Netherlands Organization for the Advancement of Pure Research (ZWO).

Up to now no suitable method has been available to assess the population density of *V. biguttatum* in soil. Because of the organism's relatively slow growth rate and low tolerance for growth retardants no successful selective culture medium could be devised. Therefore, malt peptone agar plates overgrown with mycelium of *Rhizoctonia solani*, previously introduced as "Rhizoctonia plates" (RP), so far appear to be the best selective substrate for *V. biguttatum*.

In this study two different samplers for dispersion of soil on RP were evaluated. The modified Andersen air sampler and the pellet sampler both uniformly distribute soil on RP as soil particles and as pellets, respectively.

To test the effectiveness of the two methods, samples of different soil types were serially inoculated with *V. biguttatum* by spraying conidial suspensions into the soil. Recovery of the fungus was determined one day after inoculation by dispersing soil on RP. After a ten-day incubation period at 20 °C the outgrowth from pellets and soil particles was recorded by counting colonies.

Results of the two procedures indicate that a strong correlation exists between the number of conidia introduced and the number of colonies recovered. The efficiency of recovery, however, is low, especially from soil pellets, and seems to depend on soil type. The low efficiency could not be ascribed to loss of viability of the inoculum or to losses due to inoculation procedures. Microscopic observations revealed that only part of the soil particles is in contact with hyphae of *R. solani*. It is assumed that for germination and possible subsequent development of colonies contact or near-contact is essential. The fact that efficiency of recovery is soil-dependent suggests that the complex of biotic and abiotic factors in the soil interferes with the germination process.

It is concluded that the Andersen sampler is much more effective for detection and assessment of *V. biguttatum* in soil than the pellet sampler.

M. P. DE NOOIJ and G. J. BLOOT (*Instituut voor Oecologisch Onderzoek, Afdeling Duinonderzoek "Weevers' Duin", Duinzoom 20a, 3233 EG Oostvoorne*)

Phomopsis subordinaria, a fungal disease of Plantago lanceolata in natural populations

Phomopsis subordinaria can be isolated from stalks of *Plantago lanceolata*. The infected stalks turn brown – and black later on – downward from just below the ear. The fungus produces black pycnidia, which may fuse to stromata. The diseased stalks bend sharply. The ears may die off in different stages of flowering or seed development.

In the field, the majority of the infected ears die before seed set, but yet some ears do mature. From different populations ripe ears were collected from both diseased and healthy stalks. There was a highly significant difference in the percentage black (= dead) seeds: in infected ears 62% of the seeds appeared to be black, in healthy ears this was 6%. Mean seed weight of the viable seeds in uninfected ears was higher than that of viable seeds in diseased ears. Ears from infected stalks contained less seeds per mm ear. Ear lenght was not influenced by the stalk infection.

Field observations, performed in different populations in August 1984, showed infection percentages of 0 to 60%. High percentages of infection were found in ruderal populations. In strongly infected populations the mean percentage of diseased stalks per plant appeared to be about 67%. Biomass estimations of diseased and healthy plants showed no difference in vegetative growth between plants with or without infection. The infected plants, however, produced a significantly higher number of ears. It is suggested that nutrients and energy, not invested in seed development, are used for the production of more ears instead of more vegetative biomass.

In a greenhouse experiment susceptible plants were inoculated with a spore suspension of 10^7 spores/ml by applying a drop of spore suspension to the stalk just below the ear at different stages of spike development. The stalk however, only became infected after it was also slightly damaged with a sterile needle (diameter 0.2 mm). It is suggested that snout-beetles, living in leaf-axils of *P. lanceolata*, play a role in spreading the disease. There was no difference in response between ears of different stages of flowering.

Suggestions on reallocation of nutrients and energy in infected plants, and on the role of snout-beatles in the infection process, will have to be confirmed by experiments. Investigations of variation in resistance of the host and in virulence of the pathogen in and between populations should give evidence on the population-biological relevance of the disease in relation to natural selection.

W. C. DEKKER and D. H. WIERINGA-BRANTS (*Willie Commelin Scholten Phytopathologisch Laboratorium, Javalaan 20, 3742 CP Baarn*).

Acquired resistance against tobacco mosaic virus in hypersensitive tobacco induced by components from intercellular fluid.

The induction of a hypersensitive reaction in lower leaves of "Xanthi nc"-tobacco by tobacco mosaic virus (TMV) leads to the development of both localized (LAR) and systemic acquired resistance (SAR), perceptible after superinfection with TMV. The SAR-effect can be enhanced by three extra TMV-inoculations on higher leaves at 7-day intervals.

SAR is problably induced by components transported from the inoculated leaves. These components could be present in the intercellular fluid (IF) of SAR-possessing leaves. Therefore IF was extracted from the upper uninoculated leaves by infiltration of the leaves in vacuo with water and centrifugation at 10.000 g for 30 min at 4 °C. The clear, yellowish IF was used immediately or freeze-dried at -20 °C.

IF from healthy or SAR-possessing plants was partly purified by gel filtration on a Sephadex G-50 column. Fractions were injected into the intercellular spaces of one half of each of several "Xanthi nc"-leaves. Two days after injection the whole leaves were inoculated with TMV. Ten days later a greater reduction (about 40%) in mean diameter was noticed for lesions in areas injected with some fractions of SAR-IF compared with the ones in untreated tissue. Healthy IF-fractions reduced the mean diameter by about 10%.

All fractions were examined for carbohydrate and protein content. The M.W. of proteins and glycoproteins of SAR-IF was determined by electrophoresis in a SDS-polyacrylamide slab gel. A

new glycoprotein was found after TMV-infection. Active fractions probably contain glycoproteins.

M. DE KAM (*Rijksinstituut voor onderzoek in de bos- en landschapsbouw, "De Dorschkamp", Postbus 23, 6700 AA Wageningen*)

Testing the susceptibility of poplars to Xanthomonas populi ssp. populi

Two inoculation methods for determining the susceptibility of poplar clones to *X. populi* subsp. *populi* were compared on 25 poplar clones, to ascertain which method best reflects field resistance to bacterial canker. The field resistance of 6 of the 25 clones was well known.

Per clone, 10 one-year-old plants were inoculated on fresh leaf scars in September with a suspension of *X. populi*; in May and June new plots of 10 plants per clone were sprayed with a bacterial suspension, without previous wounding. Subsequent canker formation was assessed using a scale with 6 classes ranging from 1 (resistant) to 6 (highly susceptible). In the spring inoculations the number of cankers was also assessed.

For each inoculation method the six reference clones were grouped into three classes of increasing susceptibility. The variables giving the best discrimination between the three classes were chosen by means of stepwise linear discriminant analysis. The other clones were then assigned to these susceptibility classes.

The so-called posteriori probabilities, which indicate the reliability of the conclusion, were calculated for each clone. The results showed that the method involving inculation in spring without wounding, very probably best reflects the field resistance. In this method the bacteria enter the host through the stipule scars.

B.C. VAN DAM (*Rijksinstituut voor onderzoek in de bos- en landschapsbouw "De Dorschkamp", Postbus 23, 6700 AA Wageningen*)

Are physiological races of Melampsora larici-populina present in Europe?

A joint research programme was carried out in France and The Netherlands in 1984 to ascertain the existence of physiological races of *M. larici-populina*. The poplar clones "Robusta", "Ogy", "Rap", "Isières", "Spijk", "Donk", "Ghoy", "Dorskamp", "Raspalje" and "Beaupré" were tested for their resistance to three rust provenances that had been collected in Belgium, France and The Netherlands, respectively. In Nancy and Wageningen inoculation experiments were done simultaneously on leaves on a film of water in petri dishes under identical, controlled conditions.

To assess the susceptibility of the clones, four characteristics were studied: incubation period, infection period, number of uredinia and number of uredospores produced. The Dutch and Belgian rust provenances attacked both "Robusta" and "Spijk", whereas the French rust attacked "Robusta" but not "Spijk". It was therefore concluded that there are two physiological races of *M. larici-populina* in Western Europe. Furthermore, the reaction of the other clones tested suggested that more races remain to be identified.

MEETING OF THE SECTION FOR WILD FLORA PROTECTION ON 26 OCTOBER 1983

J. VAN GROENENDAEL (*Vegetatiekunde, Plantenoecologie en Onkruidkunde LHS, Postbus 8128, 6700 ET Wageningen*)

The introduction of plant species from an ecological point of view

The attitude of man towards nature, more specially towards the plants, is essentially ambivalent. On the one hand he is a most efficient herbivore, relying on his capacity to manipulate his surroundings. On the other hand he feels reverence for the complexity and uniqueness of the living world of which he is a part. This results in conflicting "solutions" to the problem of a deteriorating environment in which many plant species are threatened, ranging from complete reconstruction of lost habitats, including plants and animals, to obstinate defense of the last remains of naturalness in nature reserves. Scientific arguments, based on the population biology of plants, may help us to understand to what extent we might expect success from our reconstruction activities, more specifically from the introduction of plant species. Two restrictions are being made: introduction is meant to include all actual introduction of seeds, plants or plant parts into a specific habitat, and not indirect conservation measures aimed at facilitation of certain species. Secondly, the species used must be native or only be extinct recently. Emphasis is put on the introduction of species in more or less natural habitats, not on the big scale planting of trees and shrubs or the sowing of grasses in recreation areas.

It is argued, that reintroduction of a species is possible only when there is a dispersal problem. This is not unlikely in a country, where more or less natural habitats become smaller and more isolated. Under this initial condition, the following points could be of interest:

1. The dispersal capacity in space of most plants is limited, for most seeds have passive- or wind-dispersal in the temperate zone, resulting in maximal dispersal distances of a few kilometres and this only with very few seeds. The dispersal in time by means of a seedbank is seldomly taken into account as a possible source for reintroduction.
2. The number of seeds used in sowing are usually far smaller when compared with the total number of seeds a plant produces over its lifetime, because that is the number a plant "uses" to re-establish a new successful individual.
3. Island biogeographical arguments might help us to establish the fact of a dispersal problem. For solving the problem, this theory probably is less useful, given the sessile character of plants. Long and narrow ribbons, connecting various elements in the landscape might be more effective than big reserves as islands in very different surroundings.
4. An introduction of a species is a success, only when there is fertile offspring from the first established plants. When the introduction is done by planting without sowing at the same time, valuable information regarding the future success of seeds might be lost.
5. Sometimes the threat to plant species is overestimated. This is the case with those species that will never be abundant, without being rare or threatened. This is also the case with species, that are characterised by rapid expansion and long periods of decline thereafter. On the other hand, many potential dominants have low dispersal capacities. Introducing those might end in overdominance or fixation of otherwise less marked phases in succession.
6. Introduction might fail because of the use of wrong ecotypes. Even when the introduction is successful, the source of the material can have its impact over long periods of time through founder-effects. When individuals of the same species are present in the neighbourhood genetic "pollution" might occur depending on relative abundances, breeding system, distance etc. Such effects are most pronounced with grasses and trees, of which cultivars are sown or planted in great quantities.

The scientific arguments used are necessary, however, not sufficient, because there is also a strong moral side to the issue. The following code of conduct is advised:

- Introduction is possible only when it is clear that there exists a major dispersal problem.
- In all cases, along with planting or without planting, seeds should be used. In sufficient quantities. The possibilities of an existing seedbank should be examined.
- Local material should be used especially with grasses and trees.

- More restrictions should be placed on introductions in areas with unique ecotypes or vegetations and close to the distribution limits of species, because of the valuable genetical and ecological information which they represent.
- The incidental success of re-introduction must not lead us to a facile belief in the possibilities of reconstruction. To protect plants and animals properly a shift in emphasis in our attitude towards nature is needed.

C.J.M. SLOET VAN OLDRUITENBORGH (*Natuurbeheer, LHS, Ritzema Bosweg 32a, 6703 AZ Wageningen*)

The naturalness of exotic (= introduced) species

Introduced species are supposed to cause severe disturbances in natural communities. Of the many thousands of species that are introduced in countries outside their original geographical area, only a small part is causing troubles, especially in more or less disturbed areas (artificial water basins, overgrazed areas, arable fields etc.).

Problems with introduced species are problems of man who meets the reactions of his own former wrong land use and introduction of species; they are not problems connected with the introduced species themselves. The first thing to do to prevent future difficulties with introduced species is therefore to forbid transportation of plants and animals outside their home-area. Two categories of organisms should be given priority: rare organisms, because of the danger of extinction (formalised in the Convention of Washington 1973) and organisms that reproduce quickly also in their own environment when disturbed.

Introduced species are not different from all other species in their basic properties: they can germinate, grow, "flower", fructifere, defend themselves, protect and adapt themselves morphologically, physiologically and genetically to changing conditions, everything to a certain extend. They do not behave differently from not introduced, indigenous species, that "take their opportunity" when possible.

Conversationists and managers of nature reserves, national parks, landscape elements etc. should rather more concentrate on stimulating the spontaneous indigenous vegetation and fauna than fight against the introduced species. Once the latter spread over an area where they are not wanted they should be considered as co-operators/selectors/regulators in arranging natural processes changing their environment and preparing it for their successors. They should be taken away at the moment they have fulfilled their role in the development of the community to make place for the indigenous organisms to take over. This means that all treatment in such areas will be on a small scale and divergent in space and time.

For the use of foreign or indigenous plants and animals in landscaping and in promoting biologically interesting communities a number of practical advices can be given:

1. use, as much as possible, the spontaneous plant growth and animal population development.
2. when planting or introduction of animals is considered inevitable: use indigenous, not foreign organisms; start with plants, not with animals; start with pioneers, adapted to the environment in case; start with seed, not with adult organisms.

G. LONDO (*Rijksinstituut voor Natuurbeheer, Postbus 46, 3956 ZR Leersum*)

Are planting and sowing useful measures in nature management?

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G. LONDO (1984): Zijn uitplanten en uitzaaien zinvolle maatregelen bij het natuurbeheer? *Levende Natuur* 85: 130-135 (with English summary).

H.J. DROST (*Rijksdienst IJsselmeerpolders, Postbus 600, 8200 AP Lelystad*)

Experiences with dissemination of wild flora species in the IJsselmeerpolders

J.J. BARKMAN (*Biologisch Station, LHS Wageningen, Kampsweg 27, 9418 PD Wijster*)

Résumé