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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Biologia vegetale. — *Callus formation and habituation in Nicotiana species in relation to the specific ability for dedifferentiation.* Nota di MARCELLO BUIATTI e ANDREA BENNICI (*), presentata (**) dal Corrisp. F. D'AMATO.

RIASSUNTO. — L'inibizione della germinazione, la formazione di callo e l'abituazione in seguito a trattamenti con 2,4-D, kinetina e IAA sono stati presi come parametri per giudicare la capacità di sdifferenziazione in *Nicotiana*.

I risultati mostrano notevoli differenze fra le 8 specie studiate (*N. bigelovii, N. glauca, N. langsdorffii, N. alata, N. debneyi, N. plumbaginifolia, N. longiflora. N. paniculata*); fra queste *N. bigelovii* ha mostrato di essere quella avente la più alta capacità di sdifferenziazione. Una ipotesi preliminare viene avanzata per spiegare i risultati in termini evolutivi.

INTRODUCTION.

Several attempts have been made in the past to give evolutionary significance to the phenomenon of formation of genetic tumors in *Nicotiana* hybrids, genetic and physiological differences have been searched for among *Nicotiana* species, which could account for their ability to dedifferentiate and give tissues autotrophic for hormones when combined in a given hybrid.

Firstly, Näf (1958) hypothesized the existence of two groups of *Nicotianae*, one formed by species of the section *Alatae* with $n = 9$ or 10 (plus group) and the other (minus group) by a variety of other species mostly with $n = 12$. According to Näf, tumors could be formed only when species of different groups were involved in the formation of a given hybrid. Later, Ahuja (1968) proposed that plus species carried genetic components essential for tumor initiation (*I*) the contribution of minus species being a genetic complex (*ee*) controlling tumor expression. At the hormone level, a generally higher endogenous auxin pool was found in (*ee*) species as compared with (*I*).

The aim of the present paper was to substantiate, if possible, these hypotheses by testing the response to hormones, as far as germination inhibition, callus formation and habituation are concerned, in 8 *Nicotiana* species belonging to different genetic phyla.

MATHERIALS AND METHODS.

Seeds of *N. bigelovii*, *N. glauca*, *N. longiflora*, *N. langsdorffii*, *N. alata*, *N. plumbaginifolia*, *N. debneyi*, *N. paniculata* were surface sterilized with 16 % commercial hypochlorite for 25 min., washed 3 times with distilled

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(**) Nella seduta del 14 febbraio 1970.

water and then sown in Erlenmeyer flasks containing 30 ml of Linsmaier and Skoog's (1965) (LS medium) basic substrate with the addition of the following hormones:

2,4–D (1×10^{-7}; 4×10^{-7}; 8×10^{-7}; 1.6×10^{-6});
kinetin (6–furfurylaminopurine) 2×10^{-7}; 4×10^{-7}; 8×10^{-7}; 1.6×10^{-6});
indoleacetic acid, (IAA) (5×10^{-7}; 1×10^{-6}; 2×10^{-6}; 4×10^{-6}).

In addition to these, the 16 possible combinations of kinetin and IAA were also tested. *p*H was kept, by NaOH or HCl adjustment, to 5.5. Experiments were done in three replicates of 40 seeds each.

Seedlings and calluses were grown in a temperature controlled room at 25° C under continuous light. Germination inhibition was scored days after sowing. Seedlings were then kept in the same conditions for another 20 days and then scored for callus formation. Callus from species which were found able to dedifferentiate was kept for 3 months on the hormone containing media and then transferred on the minimal medium (without hormones) and scored for habituation.

Results.

As to germination inhibition, 2,4–D was found to be effective on all species tested but *N. glauca*; kinetin and IAA combinations were effective on 4 species (*N. plumbaginifolia*, *N. longiflora*, *N. bigelovii*, *N. debneyi*) whereas kinetin and IAA alone did not show any significant effect.

Table I.

Germination of seeds exposed to different concentrations of 2,4–D *expressed as* % *of control, 20 days after sowing.*

Species	2,4–D concentrations in p.p.m.				
	0.1	0.2	0.4	0.8	1.6
N. glauca	102.54	89.78	77.60	99.25	89.99
N. paniculata	91.02	56.42	46.23	31.34	14.91
N. debneyi	113.26	97.94	60.47	87.29	38.01
N. bigelovii	71.88	53.88	22.08	—	19.78
N. plumbaginifolia . .	80.86	77.15	58.69	14.46	—
N. longiflora	65.13	36.63	20.93	21.14	—
N. alata	77.42	106.45	77.27	78.75	52.17

Germination of *Nicotiana* seeds, sown on media with different hormone concentrations, is reported in Table I and II. The response to 2,4–D allowed the subdivision of the 8 species tested into grossly two groups, one slightly

(*N. alata*, *N. debneyi*) and the other strongly inhibited (*N. longiflora*, *N. plumbaginifolia*, *N. bigelovii*). *N. paniculata* showed an intermediate level of inhibition, *N. glauca* did not show any inhibition at the concentrations used.

TABLE II.

Germination of seeds exposed to different concentrations of kinetin plus IAA *expressed as % of control, 20 days after sowing.*

SPECIES	Kinetin + IAA concentrations (p.p.m.)							
	0.2+0.5	0.2+1.0	0.2+2.0	0.2+4.0	0.4+0.5	0.4+1.0	0.4+2.0	0.4+4.0
N. paniculata	116.66	132.56	137.72	122.50	106.05	141.12	122.31	107.78
N. plumbaginifolia	118.90	63.69	111.48	83.92	76.43	94.43	52.46	75.09
N. debney	90.90	116.66	144.44	114.28	75.00	106.66	85.70	100.00
N. bigelovii	95.23	79.35	55.55	80.80	55.55	42.73	66.66	83.33
N. longiflora	—		104.93	95.39	113.30	120.84	72.51	72.51
N. alata	154.75	185.72	196.96	180.56	189.59	150.01	177.26	176.05
N. langsdorffii	97.22	84.84	85.55	97.22	102.08	89.74	80.21	106.94

SPECIES	Kinetin + IAA concentrations (p.p.m.)							
	0.8+0.5	0.8+1.0	0.8+2.0	0.8+4.0	1.6+0.5	1.6+1.0	1.6+2.0	1.6+4.0
N. paniculata	86.17	99.43	76.38	112.17	80.45	109.37	62.49	81.79
N. plumbaginifolia	59.45	47.55	28.14	50.30	14.85	19.28	28.14	19.10
N. debney	66.66	62.50	86.94	42.10	15.38	35.28	63.62	26.08
N. bigelovii	64.81	48.61	37.03	58.47	77.77	97.22	27.77	96.28
N. longiflora	45.32	23.38	63.95	40.28	24.98	35.06	11.67	34.51
N. alata	176.05	141.30	72.22	116.07	—	176.05	138.67	151.67
N. langsdorffii	87.50	95.44	86.41	104.16	107.33	98.24	84.26	93.33

Three out of the four species strongly inhibited by 2,4–D gave the same answer when treated with kinetin + IAA at concentrations ranging from 0.4–2.0 p.p.m. respectively, to 1.6–4.0 p.p.m. Moreover, *N. debneyi* was also inhibited although belonging to the " slight inhibition " group in the 2,4–D test. As to callus formation in presence of 2,4–D, *N. glauca*, *N. bige-*

lovii, *N. alata*, *N. plumbaginifolia*, and at a lower degree, *N. longiflora* and *N. debneyi* all formed calluses. *N. paniculata*, on the other hand, developed normal seedlings after germination.

TABLE III.

Callus formation 40 *days after sowing on media containing different* 2,4–D *concentrations.*

SPECIES	2,4–D concentration (p.p.m.)				
	0.1	0.2	0.4	0.8	1.6
N. glauca	∓	+	++	+++	+++
N. paniculata	—	—	—	—	—
N. bigelovii	++	++	+++	++	∓
N. debney	—	—	+	+	+
N. plumbaginifolia . .	∓	+	++	++	—
N. alata	+	+	+++	++	++
N. langsdorffii	∓	∓	++	—	—
N. longiflora	∓	∓	—	—	—

Legend: ∓: root thickening
+, ++, +++: increasing callus growth
—: no callus formation

A similar situation was shown by the response to kinetin and IAA combinations, (Table IV), although the number of callus forming species was restricted to 5 out of 8. In this case, *N. bigelovii* showed some dedifferentiation signs at all concentrations tested. *N. plumbaginifolia*, *N. alata*, *N. langsdorffii* and *N. longiflora* started to form callus or root thickening when exposed to 0,4 p.p.m. kinetin and 4.0 p.p.m. IAA. *N. glauca* and *N. debneyi* failed to show a similar behaviour to that found when seeds were treated with 2,4–D though the first species gave in very few seedlings some signs of dedifferentiation (expressed mainly as hypocotile thickening). Kinetin alone (Table V) induced root thickening (eventually leading to callus growth) in *N. bigelovii*, *N. plumbaginifolia*, *N. longiflora*. Finally, only *N. bigelovii* dedifferentiated when exposed to IAA given at 2 and 4 p.p.m. (Table VI).

N. glauca, *N. plumbaginifolia*, *N. alata*, *N. langsdorffii*, *N. bigelovii* calluses were then tested for habituation on a hormoneless medium. *N. alata* and *N. langsdorffii* tissues died after about 50 days of growth on LS (second

TABLE IV.

Callus formation 40 *days after sowing on media containing different kinetin* + IAA *combinations* (Legend as in Table III).

SPECIES	Kinetin + IAA concentrations (p.p.m.)							
	0.2+0.5	0.2+1.0	0.2+2.0	0.2+4.0	0.4+0.5	0.4+1.0	0.4+2.0	0.4+4.0
N. glauca	—	—	—	—	—	—	—	—
N. debney	—	—	—	—	—	—	—	—
N. paniculata	—	—	—	—	—	—	—	—
N. bigelovii	+	∓	—	—	—	∓	∓	++
N. plumbaginifolia	—	—	—	—	—	—	—	—
N. alata	—	—	—	—	—	—	—	∓
N. langsdorffii	—	—	—	—	—	—	—	∓
N. longiflora	—	—	—	—	—	—	—	—

SPECIES	Kinetin + IAA concentrations (p.p.m.)							
	0.8+0.5	0.8+1.0	0.8+2.0	0.8+4.0	1.6+0.5	1.6+1.0	1.6+2.0	1.6+4.0
N. glauca								
N. debney	—	—	—	—	—	—	—	—
N. paniculata	—	—	—	—	—			
N. bigelovii	+++	∓	∓	+	++	++	++	+++
N. plumbaginifolia	∓	∓	∓	∓	++	—	+	∓
N. alata	—	+	—	—	++	∓	+	+
N. langsdorffii	—	—	—	—	—	∓	∓	—
N. longiflora	∓	∓	∓	—	—	∓	∓	—

transfer). *N. glauca* showed regular growth for 50 days, after which most of the callus died, leaving some isolated, slow growing green nodules. *N. plumbaginifolia* callus started to differentiate leaves and roots soon after transfer in LS. Undifferentiated green tissues survived for a long time, but led eventually to more buds and roots. *N. bigelovii*, on the other hand, when grown on LS turned rapidly to a green colour, from the yellowish one present when the tissues was grown on 2,4–D, and acquired a fast growth pattern, showing little or no signs of differentiation.

TABLE V.

Callus formation 40 *days after sowing on media containing different kinetin concentrations* (Legend as in Table III).

SPECIES	Kinetin concentrations (p.p.m.)			
	0.2	0.4	0.8	1.6
N. glauca	—	—	—	—
N. debney	—	—	—	—
N. paniculata	—	—	—	—
N. bigelovii	∓	∓	∓	+
N. plumbaginifolia . .	—	∓	∓	∓
N. alata	—	—	—	—
N. langsdorffii	—	—	—	—
N. longiflora	—	∓	∓	∓

TABLE VI.

Callus formation 40 *days after sowing on media containing different* IAA *concentrations* (Legend as in Table III).

SPECIES	IAA concentrations (p.p.m.)			
	0.5	1	2	4
N. glauca	—	—	—	—
N. debney	—	—	—	—
N. paniculata	—	—	—	—
N. bigelovii	—	—	++	++
N. plumbaginifolia . .	—	—	—	—
N. alata	—	—	—	—
N. langsdorffii	—	—	—	—
N. longiflora	—	—	—	—

This behaviour was maintained, for practically 100 % of the tissue, up to present (10 months) from culture initiation. The strikingly strong ability of *N. bigelovii* to become habituated (*i.e.* autonomous for hormone requirements) led us to a further experiment on the threshold of habituation in this species. Seeds of *N. bigelovii* were sown on 2,4–D (0.1, 0.4, 0.8 p.p.m.) containing LS medium and then transferred on LS alone after 7 or 20 days.

TABLE VII.

Habituation of N. bigelovii *seedlings exposed to different concentrations of* 2,4–D.

	Control	2,4–D 0.1 p.p.m.	2,4–D 0.4 p.p.m.	2,4–D 0.8 p.p.m.
% habituated seedlings after 7 days treatment	—	25.00	33.33	50.00
% habituated seedlings after 20 days treatment	—	100.00	100.00	91.66

In Table VII, the results of such an experiment are shown. At all concentrations tested, 20 days of treatment are sufficient for dedifferentiation and habituation in 100 % of the seedlings. When seeds are treated for 7 days only, a clear dependence of the frequency of habituated seedlings on 2,4–D concentration is seen.

DISCUSSION.

In Table VIII results concerning callus formation in different species under varying hormone treatment are summarized.

The 8 species investigated can be distributed along a scale of " dedifferentiating ability ": *N. bigelovii* appears as the most unstable species, from a developmental point of view; *N. plumbaginifolia* and *N. longiflora*, both with $2n = 20$ and belonging to section *Alatae*, immediately follow. Then, two more *Alatae* species, both with $2n = 18$, can be placed at the same level on the scale. *N. debneyi*, *N. glauca*, which are able to form callus only in presence of 2,4–D come next, whilst *N. paniculata*, which never dedifferentiates, lies at the very end of the scale. None of the last species belongs to the section *Alatae* and only *N. debneyi* is included, according to Goodspeed (1954) in the Subgenus *Petunioides*.

TABLE VIII.

Comprehensive picture of callus formation in Nicotiana *species, exposed to different hormone treatments (in parenthesis, signs of Näf's classification).*

	N. bigelovii (—)	N. plumbaginifolia (+)	N. longiflora (+)	N. alata (+)	N. langsdorffii (+)	N. debneyi (—)	N. glauca (—)
IAA . .	+	—	—	—	—	—	—
Kinetin .	+	+	+	—	—	—	—
Kinetin+							
IAA . .	+	+	+	+	+	—	—
2,4–D . .	+	+	+	+	+	+	+

If the scale just drawn is considered in terms of plus and minus species (Näf 1954) or of (*I*) and (*ee*) species (Ahuja 1968) we find that 4 out of the first five species in the scale are classified as « plus »; on the other hand, the three remaining species belong to the minus group; the only relevant exception to this rule is *N. bigelovii*. This species though being " minus ", behaves as " plus ", when crossed with *N. glauca*. This exception is explained by Näf by assuming a different " strength " in contributing to tumor formation within " plus " and " minus " species. If, however, attention is drawn to the phylogenetic situation of the species used in the present study (fig. 1), one can easily see that the ability to form callus decreases the further we move from *N. bigelovii*.

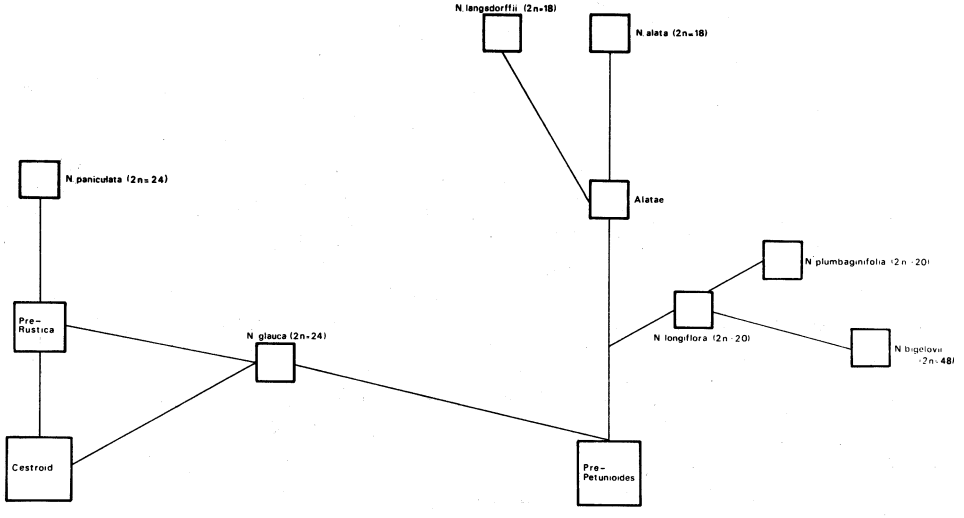

Fig. 1.

On the basis of the other results of previous workers and our own, we can tentatively hypothesize the isolation, through evolution, of a number of genes responsible for tumor induction (the inducer (I) genes of Ahuja) in the Subgenus *Petunioides* which find optimal expression in the Sections *Alatae* and *Bigelovianae*. This hypothesis should, of course, be tested by using *N. clevelandii* which is also classified by Goodspeed (1954) in the Section *Bigelovianae* as well as other varieties of *N. bigelovii* itself, in the appropriate crosses. The data on germination inhibition grossly showed the same pattern as the callus formation experiments; a greater inhibition was found in *N. longiflora*, *N. bigelovii* and *N. plumbaginifolia* (these species, according to our hypothesis, carry *I* genes); conversely, the only species which never showed inhibition was *N. glauca*, which, according to Näf and to our results on callus formation, is regarded as a " minus " species.

The results are somewhat more confused for the species showing intermediate levels of inhibition.

The interpretation of the forementioned results in terms of internal hormone level of the species tested is, however, very difficult. This is in contrast with analyses carried out by several authors (Ahuja and Hagen 1966, Bayer 1967, Bayer and Ahuja, 1968) on the auxin-auxininhibitor levels in *Nicotiana* species which point to a higher level in minus species (carriers of *ee* genes).

Inhibition data would best be accounted for by higher hormone synthetic ability in plus species, thus lowering the inhibition threshold.

Finally, a word should be spent on the particularly interesting results obtained with *N. bigelovii*. This species seems one of the most unstable ones yet found, because it is habituated efficiently after a very short hormone treatment, whilst habituation usually appears in small tissue amounts and after very long periods of culture. Another exception to this general rule seems to be *Lilium longiflorum* which, according to Sheridan (1968), becomes autotrophic after a short period of growth on an IAA containing medium. These two species, hence, seem to provide a particularly interesting material for studies on the biochemistry and biology of habituation (transformation to autotrophy) in plant material.

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