Detection of Four Lethality Types in Interspecific Crosses among *Nicotiana* Species through the Use of Three Rescue Methods for Lethality

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Summary

Hybrid lethality was surveyed in 186 cross combinations among 17 species from 9 sections of the genus *Nicotiana*. The hybrid seedlings of 14 cross combinations expressed lethality characterized by one of four symptoms and the lethality was classified into four types as follows: browning of the shoot apex and the root tip (Type I), browning of the hypocotyl and the roots (Type II), yellowing of the true leaves (Type III) and formation of multiple shoots (Type IV). Three kinds of rescue methods for the lethality were applied to the 4 cross combinations expressing different types of the lethality as follows: (1) culture of hybrid seedlings under high temperature conditions to suppress the lethality, (2) culture of the cotyledonary segments of the hybrid seedlings to obtain regenerants overcoming the lethality and (3) culture of the hybrid seedlings on a medium containing cytokinin to obtain regenerants overcoming the lethality. The 1st and 2nd methods were effective in the cross of *N. nudicaulis* × *N. tabacum* expressing the lethality Type I. All the methods were effective in the cross of *N. paniculata* × *N. suaveolens* expressing the lethality Type II. Only the 1st method was effective in the cross of *N. glutinosa* × *N. repanda* expressing the lethality Type III. None of the methods were effective in the cross of *N. paniculata* × *N. alata* expressing the lethality Type IV. Each lethality type exhibited a specific set of responses to the three rescue methods. These findings suggest that the lethality observed in interspecific crosses of *Nicotiana* differs in the physiological processes leading to death.

Key Words : genus *Nicotiana*, cotyledon culture, cytokinin, high temperature, hybrid lethality, interspecific hybrid.

Introduction

Hybrid lethality is one of the sexual barriers preventing wide hybridization. Some cross combinations expressing the lethality have been reported in the interspecific hybrids of several genera, such as *Triticum* (Chen *et al.* 1989), *Oryza* (Oka 1962) and *Gossypium* (Philips 1977).

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In the genus *Nicotiana*, Kostoff (1930) performed the hybridization of 80 cross combinations among 16 species, and observed that the hybrids of 9 cross combinations died at the seedling stage. De Verna *et al.* (1987) performed 10 reciprocal crosses between *N. tabacum* cv. 'Ky 17' and 5 wild species in *Nicotiana* using *in vitro* pollination and ovule culture, and reported that the seedlings derived from 2 crosses always displayed lethal necrosis. However, the lethal symptoms displayed by these hybrid seedlings were not described in detail.

It is important to overcome the lethality in interspecific hybrids to widen genetic variation in cultivated species. So far, rescue methods for the lethality have been developed in a few cross combinations of the *Nicotiana* species. For example, in the cross of *N. suaveolens* × *N. tabacum*, three rescue methods have been developed. Marubashi *et al.* (1988) reported that the lethality was suppressed by culturing the hybrid seedlings under high temperature conditions (36 °C). Lloyd (1975) cultured cotyledonary segments of the hybrid seedlings on media containing plant growth regulators and obtained viable hybrids from regenerants through the callus. Inoue *et al.* (1994, 1997) cultured the intact hybrid seedlings on media containing cytokinins and obtained viable hybrids derived from adventitious buds that were regenerated from the hypocotyl of dying hybrid plants. Through the use of these three rescue methods, it was anticipated that the physiological processes involved in the lethality could be elucidated along with the primary objective of rescuing the hybrids from lethality.

In this study, we surveyed the cross combinations expressing lethality among the *Nicotiana* species and classified the lethality based on the symptoms. Furthermore, we applied three rescue methods to the hybrid seedlings showing different lethal symptoms and attempted to analyze the physiological characteristics of lethality in interspecific crosses of the genus *Nicotiana*.

Materials and Methods

Plant materials

Seventeen species of *Nicotiana* were used in this experiment (Table 1). The seeds of these species were supplied by Japan Tobacco Inc. and maintained in our laboratory. Plants were grown in a greenhouse of the School of Agriculture, Ibaraki University from February to December in 1994.

Table 1. *Nicotiana* species used in the interspecific hybridization

Section ¹⁾	Species	Haploid chromosome numbers ¹⁾
<i>Paniculatae</i>	<i>N. glauca</i>	12
	<i>N. paniculata</i>	12
<i>Rusticae</i>	<i>N. rustica</i>	24
<i>Tomentosae</i>	<i>N. glutinosa</i>	12
	<i>N. otophora</i>	12
<i>Genuinae</i>	<i>N. tabacum</i>	24
<i>Trigonophyllae</i>	<i>N. trigonophylla</i>	12
<i>Alatae</i>	<i>N. alata</i>	9
	<i>N. longiflora</i>	10
	<i>N. plumbaginifolia</i>	10
	<i>N. sylvestris</i>	12
<i>Repandae</i>	<i>N. repanda</i>	24
<i>Nudicaules</i>	<i>N. nudicaulis</i>	24
<i>Suaveolentes</i>	<i>N. suaveolens</i>	16
	<i>N. gossei</i>	19
	<i>N. megalosiphon</i>	20
	<i>N. debney</i>	24

¹⁾ According to the description of Goodspeed (1954).

Cross-pollination

Cross-pollination was performed reciprocally between two species whose flowering time was compatible. Flowers of the maternal species, which were emasculated before flowering, were pollinated with fresh pollen of the paternal species. Thus, 186 cross-pollinations were successful among 17 species.

Germination of hybrid seeds

Ten F₁ seeds for each successful cross were soaked in a 0.5% Gibberellin A₃ solution for 30 minutes, sterilized with a 5% sodium hypochlorite solution (0.25% liberated chloride) for 15 minutes and rinsed three times with sterilized water. F₁ seeds were sown on MS medium (Murashige and Skoog 1962), with half the concentration of salts, supplemented with 1% sucrose and 0.2% gelrite (pH 5.8), and were cultured at 28 °C under continuous illumination (ca. 3,000 lx) for germination. Under these conditions, lethality was expressed in the crosses of *N. suaveolens* × *N. tabacum* (Manabe *et al.* 1989). Lethal symptoms were investigated in the hybrids throughout the growth period. Surviving seedlings were transferred from the medium to soil at 60 days after germination.

Culture under high temperature conditions to suppress the lethality-high temperature culture

Germinating hybrids were exposed to six different temperatures (28, 30, 32, 34, 36, 38 °C) under continuous illumination (ca. 3,000 lx). All the seedlings were

observed for the expression of lethality after the initiation of high temperature culture. At 45 days after germination, the surviving seedlings were transferred from the medium to soil in pots. Half of them grew at the same temperature and the others were exposed to the lethal temperature of 28 °C to confirm the suppression of the lethality by the high temperature conditions (Manabe *et al.* 1989).

Culture of cotyledonary segments to overcome the lethality-cotyledon culture

Cotyledon culture was performed according to the method of Lloyd (1975). A cotyledon of the hybrid seedling was cut into four transverse segments 2–3 mm in width. The segments were placed on MS medium, supplemented with 2 mg l⁻¹ of α -naphthalenacetic acid (NAA), 2 mg l⁻¹ of 6-benzylaminopurine (BAP), 3% sucrose and 0.8% agar (pH 5.5), and cultured at 28 °C under continuous illumination (ca. 3,000 lx) for dedifferentiation and shoot formation. For rooting, the regenerated shoots were transferred to MS medium, supplemented with 2 mg l⁻¹ NAA, 3% sucrose and 0.8% agar (pH 5.5).

Culture of hybrid seedlings on the medium containing cytokinin to overcome the lethality-cytokinin culture

Cytokinin culture was performed according to the method of Inoue *et al.* (1994). The hybrid seedlings were transferred to MS medium, with half the concentration of salts, supplemented with 0–10 mg l⁻¹ BAP, 1% sucrose and 0.2% gelrite (pH 5.8), and were cultured at 28 °C under continuous illumination (ca. 3,000 lx). For rooting, the adventitious shoots formed on the seedlings were cut and transferred to MS medium, with half the concentration of salts, supplemented with 2 mg l⁻¹ NAA, 1% sucrose and 0.2% gelrite (pH 5.8).

Chromosome observation

In the above two methods for overcoming lethality, all the surviving regenerants were transferred from the medium to soil and were grown to maturity in a greenhouse. Chromosomes were stained with a basic fuchsin solution and were counted under a light microscope.

Results

Survey of lethality

Pod formation was observed in 108 of the 186 cross combinations performed. Germination was observed in 61 cross combinations. The hybrid seedlings from 14 cross combinations expressed the lethality and died within 60 days after germination, whereas, no lethal symptoms were observed in the other 47 cross combinations.

Four lethality types were detected in the above 14 cross combinations based on the lethal symptoms of the seedlings by which the cross combinations were clas-

Table 2. Lethality types detected in the interspecific crosses of *Nicotiana*

Lethality type	Lethal symptoms ¹⁾	Cross combinations ²⁾
Type I	Browning of shoot apex and root tip	<i>N. nudicaulis</i> × <i>N. tabacum</i>
Type II	Browning of hypocotyl and roots	<i>N. paniculata</i> × <i>N. gossei</i> <i>N. paniculata</i> × <i>N. suaveolens</i> <i>N. debneyi</i> × <i>N. tabacum</i> <i>N. debneyi</i> × <i>N. sylvestris</i> <i>N. debneyi</i> × <i>N. repanda</i>
Type III	Yellowing of true leaves	<i>N. paniculata</i> × <i>N. nudicaulis</i> <i>N. glutinosa</i> × <i>N. repanda</i> <i>N. trigonophylla</i> × <i>N. otophora</i> <i>N. megalosiphon</i> × <i>N. longiflora</i> <i>N. megalosiphon</i> × <i>N. plumbaginifolia</i>
Type IV	Formation of multiple shoots	<i>N. paniculata</i> × <i>N. glutinosa</i> <i>N. paniculata</i> × <i>N. alata</i> <i>N. gossei</i> × <i>N. alata</i>

¹⁾ All the symptoms were observed in the hybrid seedlings by 45 days after germination.

²⁾ All the seedlings which germinated showed the lethal symptoms.

Fig. 1. Lethal symptoms observed in the hybrid seedlings of the *Nicotiana* cross combinations: a, browning of shoot apex and root tip at the time of germination in the cross *N. nudicaulis* × *N. tabacum*; b, browning of hypocotyl and roots one week after germination in the cross *N. paniculata* × *N. suaveolens*; c, yellowing of true leaves at 3 weeks after germination in the cross *N. glutinosa* × *N. repanda*; d, formation of multiple shoots at 3 weeks after germination in the cross *N. paniculata* × *N. alata*.

sified (Table 2). In the cross of *N. nudicaulis* × *N. tabacum*, the hybrid seedlings showed browning of the shoot apex and the root tip (Fig. 1a). Lethality characterized by these symptoms was not detected in any of the other combinations and was referred to as Type I. In the cross of *N. paniculata* × *N. suaveolens*, the hybrid seedlings showed browning of the hypocotyl and the roots (Fig. 1b). Lethality characterized by these symptoms was detected in the 5 combinations and referred to as Type II. In the cross of *N. glutinosa* × *N. repanda*, the hybrid seedlings showed yellowing of the true leaves (Fig. 1c). Lethality associated with this symptom was detected in the 5 combinations and referred to as Type III. In the cross of *N. paniculata* × *N. alata*, the multiple shoots were formed on the basal part of the seedlings (Fig. 1d) and underwent browning. Lethality characterized by this symptom was detected in the three combinations and referred to as Type IV.

In order to analyze the physiological characteristics of the lethality, 3 rescue methods were applied to 1 cross combination from each lethality type; i. e. *N. nudicaulis* × *N. tabacum* (Type I), *N. paniculata* × *N. suaveolens* (Type II), *N. glutinosa* × *N. repanda* (Type III) and *N. paniculata* × *N. alata* (Type IV). These cross combinations were selected for their high seed setting and high germination rate.

Effect of high temperature culture

The hybrid seedlings of the above 4 cross combinations showed different responses under the various temperature conditions (Table 3). Hybrid seedlings of *N. nudicaulis* × *N. tabacum* (Type I) and *N. paniculata* × *N. suaveolens* (Type II) expressed the lethality at 28 and 30 °C but not at 32–38 °C (Fig. 2a, b). Hybrid seedlings of *N. glutinosa* × *N. repanda* (Type III) died within 30

Table 3. Effect of temperature conditions on the growth of hybrid seedlings derived from four *Nicotiana* crosses

Cross combination	Culture temperature (°C)	No. of F ₁ seedlings cultured	No. of surviving seedlings	
			30 ¹⁾	45
<i>N. nudicaulis</i> × <i>N. tabacum</i>	28	10	0	0
	30	10	0	0
	32	16	16	16
	34	18	18	18
	36	10	10	10
	38	15	15	15
<i>N. paniculata</i> × <i>N. suaveolens</i>	28	16	0	0
	30	14	0	0
	32	14	14	14
	34	16	16	16
	36	15	15	15
	38	16	16	16
<i>N. glutinosa</i> × <i>N. repanda</i>	28	10	0	0
	30	10	0	0
	32	10	6	6
	34	10	0	0
	36	10	0	0
	38	10	0	0
<i>N. paniculata</i> × <i>N. alata</i>	28	10	4	0
	30	10	6	0
	32	10	10	0
	34	10	6	0
	36	10	7	0
	38	10	0	0

¹⁾ Days after germination.

days at 28–30 °C and showed chlorosis and stopped growing after 30 days at 34–38 °C (Fig. 2c). In this combination at 32 °C, hybrid seedlings showed a delay in the appearance of the lethal symptoms and survived for 45 days after germination. In the cross of *N. paniculata* × *N. alata* (Type IV), some of the 10 hybrid seedlings expressed the lethality within 30 days after germination at all the temperatures except for 32 °C. All the hybrid seedlings, including hybrid plants which did not display lethal symptoms at first, showed necrosis and died within 45 days after germination.

Hybrid seedlings of *N. nudicaulis* × *N. tabacum* and *N. paniculata* × *N. suaveolens* grew to maturity under the high temperature conditions (32–38°C). When exposed to 28°C, they died after showing the typical symptoms.

Effect of cotyledon culture

In the 4 cross combinations, the cotyledonary segments behaved differently on the medium for dedifferentiation and shoot formation (Table 4). In the cross of *N. nudicaulis* × *N. tabacum*, all the segments showed browning and no further growth. In the cross of *N.*

paniculata × *N. suaveolens*, 15 of the 27 segments formed calli and many vigorous shoots were regenerated from the surface of the calli (Fig. 3a, b). In the cross of *N. glutinosa* × *N. repanda*, 3 of the 25 segments formed calli and some shoots were regenerated. These shoots showed chlorosis and did not grow further. In the cross of *N. paniculata* × *N. alata*, 8 of the 10 segments formed calli. These calli showed browning and failed to grow.

In the cross of *N. paniculata* × *N. suaveolens*, vigorous shoots regenerated roots on the rooting medium (Fig. 3c). The regenerants showed vigorous growth and became mature (Fig. 3d). The chromosome number of the somatic cells was 28, which corresponded to the combined number of the parental species. Cotyledon culture was effective in overcoming the lethality in this cross combination.

Effect of cytokinin culture

When cultured on the media containing cytokinin (BAP) at various concentrations, the hybrid seedlings derived from the 4 cross combinations showed different reactions after expressing the lethality (Table 5). In the cross of *N. nudicaulis* × *N. tabacum*, the seedlings showing

Fig. 2. Hybrid seedlings cultured at various temperatures at 30 days after germination: a, cross *N. nudicaulis* × *N. tabacum*; b, cross *N. paniculata* × *N. suaveolens*; c, cross *N. glutinosa* × *N. repanda*.

Table 4. Growth of callus and shoots from the cotyledonary segments of hybrid seedlings derived from four *Nicotiana* crosses

Cross combination	No. of segments cultured	No. of segments with ¹⁾		
		only callus	callus+ shoots	no response
<i>N. nudicaulis</i> × <i>N. tabacum</i>	25	0	0	25
<i>N. paniculata</i> × <i>N. suaveolens</i>	27	0	15	12
<i>N. glutinosa</i> × <i>N. repanda</i>	25	0	3	22
<i>N. paniculata</i> × <i>N. alata</i>	10	8	0	2

¹⁾ The data were obtained 30 days after the initiation of culture.

symptoms of the lethality formed calli from the browning hypocotyl on the media containing 0.2 or 2 mg l^{-1} BAP (Fig. 4a). On the medium containing 0.2 mg l^{-1} BAP, the calli regenerated numerous vigorous shoots. In the cross of *N. paniculata* × *N. suaveolens*, the seedlings showing symptoms of the lethality regenerated numerous vigorous shoots directly from the browning hypocotyl on the media containing 0.2–10 mg l^{-1} BAP (Fig. 4b). In the cross of *N. glutinosa* × *N. repanda*, the seedlings showing symptoms of the lethality formed calli from the root tip on the media containing 2 or 10

Fig. 3. Raising process of surviving hybrid plants through culture of the cotyledonary segment of a hybrid seedling in the cross *N. nudicaulis* × *N. tabacum*: a, callus formed from a cotyledonary segment at 7 days after initiation of the culture; b, multiple shoots redifferentiated from the callus at 30 days after initiation of the culture; c, vigorous plant regenerated from the shoot at 60 days after initiation of the culture; d, mature plant at 4 month after initiation of the culture.

mg l^{-1} BAP. On the medium containing 10 mg l^{-1} BAP, the calli regenerated shoots. These shoots showed chlorosis and did not grow further. In the cross of *N. paniculata* × *N. alata*, the seedlings exhibiting chlorosis formed calli from the hypocotyl on the media containing 0.2–10 mg l^{-1} BAP. All the calli showed browning and did not grow further.

The vigorous shoots of *N. nudicaulis* × *N. tabacum*, *N. glutinosa* × *N. repanda* regenerated roots on the medium for rooting. These regenerants displayed vigorous growth and matured. The chromosome numbers of the somatic cells were 28 and 48 respectively, which corresponded to the combined number of the parental species. Culture on the media containing cytokinin was effective in overcoming the lethality in these 2 cross combinations.

Classification of lethality

Three rescue methods showed different effects on the four lethality types (Table 6). The lethality types were classified according to the responses of the hybrid seedlings to the rescue methods. In the case of lethality Type I, high temperature conditions and cytokinin culture exerted beneficial effects unlike cotyledon cul-

Table 5. Effect of cytokinin on callus or shoot formation in hybrid seedlings derived from four *Nicotiana* crosses

Cross combination	BAP ¹⁾ concentration (m/l)	No. of seedlings cultured	No. of seedlings with ²⁾			
			only callus	callus+ shoots	only shoots	no response
<i>N. nudicaulis</i> × <i>N. tabacum</i>	0	8	0	0	0	8
	0.01	9	0	0	0	9
	0.2	11	0	11	0	0
	2	10	7	0	0	3
	10	6	0	0	0	6
<i>N. paniculata</i> × <i>N. suaveolens</i>	0	8	0	0	0	8
	0.01	8	0	0	0	8
	0.2	9	0	0	8	1
	2	6	0	0	5	1
	10	6	0	0	4	2
<i>N. glutinosa</i> × <i>N. repanda</i>	0	15	0	0	0	15
	0.01	15	0	0	0	15
	0.2	15	0	0	0	15
	2	15	15	0	0	0
	10	11	9	2	0	0
<i>N. paniculata</i> × <i>N. alata</i>	0	4	0	0	0	4
	0.01	5	0	0	0	5
	0.2	3	2	0	0	1
	2	4	1	0	0	3
	10	1	1	0	0	0

¹⁾ 6-benzylaminopurine.

²⁾ The data were obtained 60 days after the initiation of culture.

Fig. 4. Formation of vigorous shoots and calli through the culture of hybrid seedlings on the media containing cytokinin (BAP) of 60 days after initiation of the culture: a, cross *N. nudicaulis* × *N. tabacum*; b, cross *N. paniculata* × *N. suaveolens*.

ture. All the methods exerted beneficial effects in the case of lethality Type II. In the case of lethality Type III, high temperature conditions suppressed the lethality but other methods did not have a beneficial effect. None the methods were effective in the case of lethality Type IV. The set of responses to the three rescue methods was specific to the four lethality types.

Discussion

Hybrid lethality is one of the constraints to be overcome in plant breeding. In this study, we characterized the lethality expressed in *Nicotiana* crosses. Fourteen cross combinations expressing the lethality were detected in 186 cross combinations of the *Nicotiana* species from 9 sections. The cross combinations were classified into four types based on the lethal symptoms. Through the use of three rescue methods, different effects were observed in each lethality type. Moreover, each lethality type showed a specific set of responses to the three rescue methods.

The lethality that had been previously reported in detail in the *Nicotiana* crosses corresponded to the four types observed in this study. From the description of the lethal symptoms in the previous studies, lethality expressed in the 2 crosses of *N. suaveolens* × *N. tabacum*

Table 6. Response to three rescue methods in hybrid seedlings

Cross combination	Lethality type	Response to		
		high temperature	cotyledon culture	cytokinin culture
<i>N. nudicaulis</i> × <i>N. tabacum</i>	I	suppressing ¹⁾	no effect ²⁾	overcoming ³⁾
<i>N. paniculata</i> × <i>N. suaveolens</i>	II	suppressing	overcoming	overcoming
<i>N. glutinosa</i> × <i>N. repanda</i>	III	suppressing	no effect	no effect
<i>N. paniculata</i> × <i>N. alata</i>	IV	no effect	no effect	no effect

¹⁾ Seedlings had been kept continuously under high temperature conditions.

²⁾ No viable seedlings or regenerated plants were obtained.

³⁾ Regenerated plants were obtained.

(Lloyd 1975, Marubashi *et al.* 1988) and *N. suaveolens* × *N. sylvestris* (Inoue *et al.* 1996) was identified as Type II. Similarly, lethality expressed in the cross of *N. repanda* × *N. tabacum* (Iwai *et al.* 1984) was identified as Type III. Type II has been detected in 8 cross combinations and Type III in 6 cross combinations including the findings in a previous study.

Type I and IV had not been reported and were detected in a few cross combinations (Type I; 1 cross combination, Type IV; 3 cross combinations) in our survey. Type I was expressed between *Genuinae* and *Nudicaules* at the level of sections (Table 1). These two sections include only one species respectively; *i. e.* *N. tabacum* and *N. nudicaulis* (Goodspeed 1954). This type may be restricted to the combination of these two sections. In Type IV, most of the seedlings formed multiple shoots and underwent gradual browning, together with the shoots. However, a few seedlings were able to grow without multiple shoots, but they also underwent browning and died within 40 days after germination. Thus, we detected new types of lethality expressed in the *Nicotiana* crosses.

According to the previous studies in 2 cross combinations (*N. suaveolens* × *N. tabacum*, *N. repanda* × *N. tabacum*) expressing the lethality, the responses to the rescue methods of the seedlings were in agreement with our results for the same lethality type shown in Table 6, respectively. In the cross of *N. suaveolens* × *N. tabacum* expressing the lethality Type II, high temperature conditions (Manabe *et al.* 1989), cotyledon culture (Lloyd 1975) and cytokinin culture (Inoue *et al.* 1994) exerted beneficial effects in the hybrid. In the cross of *N. repanda* × *N. tabacum* expressing lethality Type III, high temperature conditions had beneficial effects (Suda and Marubashi 1990), unlike cotyledon culture (Iwai *et al.* 1984) in the hybrid. Therefore, it is suggested that the responses to the rescue methods are specific to each lethality type.

In this study, we carried out morphological and physiological characterization of the lethality and detected four lethality types in the interspecific crosses of *Nicotiana*. It is suggested that the lethality observed in the *Nicotiana* crosses undergoes different physiological

processes leading to death.

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