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Pollen Viability Assessments in Blackberries (*Rubus* subgen. *Rubus*)

By

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Abstract: Pollen viability has been investigated in 20 blackberry species using 3 methods, (1) cotton blue, (2) TTC, and (3) germination in a sucrose solution. Significant differences were found between species. Correlations between high pollen viability and high seed set were also obtained.

Polyploid blackberries are facultatively apomictic but also pseudogamous, i.e. the parthenogenic embryo development is dependent on prior pollination. Pollen viability is thus of importance for successful seed set. However, several authors have reported severe meiotic disturbances in pollen meiosis in polyploid blackberry species; an exhaustive account on Swedish species is given by GUSTAFSSON (1943), who also reports low pollen viability as deduced from cotton blue staining. An average of only 8% good pollen was obtained for triploids and 54% for tetraploids as compared with 81% for the amphimictic diploids. The taxa in the critical *Corylifolius* group are usually tetra-, penta- or hexaploid with an average pollen stainability of 23%, 69% and 75% respectively (GUSTAFSSON 1943). These taxa are usually regarded as hybrids between other blackberries and the tetraploid dewberry (*R. caesius*), which has fairly regular meiosis and 90–100% good pollen. VIRDI & al. (1972) working with raspberry cultivars (*R. idaeus,* diploid amphimict) report an association between meiotic irregularities and reduced pollen fertility as deduced from acetocarmine staining.

A series of experiments to assess the importance of pollen donor identity, pollen quality and various pollination mechanisms (the occur-

rence of active self-pollination in some species, NYBOM 1985) for seed set and the proportion offspring produced by apomixis and amphimixis respectively, was carried out on up to 20 Swedish polyploid blackberry species. As part of this work, three methods for assessing pollen viability were tested: cotton blue (aniline blue lactophenol), TTC (triphenyltetrazoliumchloride) and pollen germination. Cotton blue stains all pollen grains that contain starch, TTC stains all enzymatically active pollen grains and the germination test assesses the proportion of pollen grains germinating in a sucrose solution.

Material

The collection of indigenous blackberry species in the Helsingborg Botanical Garden (Fredriksdal) in S Sweden comprises c. 20 taxa (the often cultivated central European *R. laciniatus* is also included), each represented by one clone. Pollen from these species was tested with cotton blue in 1983 (NYBOM 1985) and three pollen viability tests were carried out in 1984 (Tab. 1).

The inflorescences were isolated in glassine bags 2–3 days before pollen was collected. This prevents contamination and increases the amount of available pollen. Pollen was obtained by gently rubbing the flower on a glass slide (for germination tests a coverslip instead). Only secondary flowers (arising from first-order laterals in the inflorescence) were used.

In 1983 slides were prepared from 10 flowers of each species twice, at intervals of 2–5 days. Flowering time does not always overlap in the different species so that pollen had to be collected on a total of 5 occasions (Tab. 3).

In 1984 the species were investigated 2–5 times each (one species once only). In all, pollen was collected 7 times at intervals of 2–4 days (Tabs. 4 and 5). Each time 10 flowers of each species were used for each test. Where possible flowers were used in one test only. For some few-flowering species the flowers had to be used in two, seldom three, tests.

In 1983 artificial self-pollination (strictly speaking within-clone pollination) was carried out using on an average 59 flowers from each species. The flower buds were emasculated 2–3 days before anthesis and isolated in glassine bags, and the pollen donor flowers were also isolated. Pollination was carried out by brushing the donor flower against the recipient flower. Only secondary flowers were used. When the drupelets had swelled sufficiently, usually 4–6 weeks after pollination, the berries were harvested and the number of well-developed drupelets in each berry counted. A corresponding set of berries from open-pollinated flowers were also harvested and their drupelets counted.

For each species 12 secondary flowers were fixed in FAA and the number of styles counted.

Of the Swedish blackberry species grown in the Lund University Botanical Garden, S Sweden, 15 species were used in this investigation, all being represented also in the Helsingborg material. Two bushes of most species were available though sometimes of different origin. Ten flowers from each bush were tested twice with cotton blue in 1984. Altogether pollen was collected 9 times at intervals of 2–12 days. For this study only averages for each species have been used.

Herbarium voucher specimens have been deposited in herb. LD.

Methods

Cotton blue was applied to pollen on a slide, a coverslip was immediately added and the slide stored in a refrigerator. Several weeks later 2 × 100 grains were counted on each slide.

The TTC solution was obtained from LINUS SVENSSON (unpublished recipe: 100 ml H_2O, 1.00 g 2,3,5-triphenyltetrazoliumchloride, 0.02 g 5,5-diethylbarbituric acid sodium salt, 1.78 g sodium succinate, 1.00 g $Ca(NO_3)_2 \cdot H_2O$, 1.82 g trishydroxymethylaminomethane, pH adjusted to 7.1 with IN HCl). Pollen grains that contain active succinic dehydrogenase are coloured red. After applying the solution a coverslip was immediately added, the slide placed in a moisture chamber and kept in daylight at room temperature for 2–4 hours, which seemed more effective for this material than incubating the slides at high temperatures in the dark as recommended by SVENSSON. 2 × 100 grains were counted on each slide immediately after incubation. Grains that stained a light pinkish colour were regarded as active.

The germination medium consisted of 20% sucrose and 50 ppm boric acid in distilled water. The medium was added to the pollen grains on coverslips which were placed in moisture chambers and incubated in artificial light at room temperature for 20–25 hours after which a drop of acetocarmine was added and the coverslip placed on a slide. 5 × 100 grains were counted on each slide. Only well-developed tubes were counted.

All slides prepared in 1983 were counted by the same person as were all slides from the Helsingborg material in 1984. The Lund material was counted by a different person.

Results

Mean values for percentage good pollen for each year from the pollen viability tests are given in Tab. 1. Correlation tests on the cotton blue values for the different species were all highly significant, both between years and between the two localities (Tab. 2). High correlation was also found between the cotton blue and TTC values for the Helsingborg material in 1984 (Tab. 2). The germination test was not significantly correlated with the two staining tests, but the values for p lie only slightly above the 0.05 limit (Tab. 2).

Mean values and standard deviations for the different collection dates are given for cotton blue and TTC for the Helsingborg material (Tabs. 3–5).

Seed set after self-pollination and after open pollination is given in Tab. 1. For some species seed set is much higher after open pollination, which may be the result of decreased self-compatibility, poor pollen viability (thus benefitting from the opportunity to obtain pollen from other species) or the handling entailed in self-pollination. The average number of styles per flower is also given in Tab. 1.

Seed set is no doubt influenced by the quality of both maternal and paternal gametes (EATON 1968). A crude way of ranking the pollen viability tests would thus be to look for correlations between percentage

Table 1. Average percentage good pollen as assessed by 3 tests. On the Helsingborg drupelets per berry after self-pollination and after open pollination respectively,

Species	Chromosome number	Helsingborg 1983 Cotton blue % good pollen	1984 Cotton blue % good pollen
R. nessensis W. HALL	2n = 28	41	47
R. scissus W. C. R. WATSON	2n = 28	34	55
R. plicatus WEIHE & NEES	2n = 28	33	34
R. sulcatus TRATT.	2n = 28	33	32
R. nitidus WEIHE & NEES	2n = 21	6	3
R. affinis WEIHE & NEES	2n = 28	48	53
R. axillaris LEJ.	2n = 28	21	–
R. insularis F. ARESCH.	2n = 28	28	25
R. lindebergii P. J. MUELLER	2n = 28	14	21
R. laciniatus WILLD.	2n = 28	23	–
R. sprengelii WEIHE	2n = 28	25	32
R. pyramidalis KALTENB.	2n = 28	24	–
R. polyanthemus LINDEB.	2n = 28	41	56
R. thyrsanthus FOCKE	2n = 21	11	12
R. bellardii WEIHE & NEES	2n = 35	43	40
R. hartmannii SUDRE	2n = 28	49	31
R. radula WEIHE & BOENN.	2n = 28	30	–
R. taeniarum LINDEB.	2n = 28	41	38
R. vestitus WEIHE & NEES	2n = 28	40	–
R. wahlbergii ARRH.	2n = 35	53	54

good pollen and relative seed set, i.e. the average number of drupelets per berry divided by the average number of styles (Tab. 6).

Another method of ranking the pollen viability tests is to investigate their ability to distinguish the species. This was done by applying parametric analyses of variance (Tab. 7). However, the pollen germination data did not meet the requirements for parametric tests and thus a non-parametric Kruskal-Wallis analysis of variance was also applied. To estimate the differences between single species pairs I also used Scheffe's a posteriori test. One-way analyses of variance were carried out on the Helsingborg values of 1984 for (1) the 15 species and (2) for those 8 species from which the pollen was collected on the same two occasions, thus diminishing the temporal influence. Finally a two-way analysis of variance was applied on these 8 species with time and species as the two factors.

Discussion

All species investigated showed fairly low cotton blue stainability which agrees well with values reported by GUSTAFSSON (1943). The

material in 1983 also average number of styles per flower and average number of have been calculated. Chromosome numbers according to GUSTAFSSON (1943)

1984 TTC % good pollen	1984 % pollen germination	Lund 1984 Cotton blue % good pollen	Number of styles	Number of drupelets per berry after self- poll.	Number of drupelets per berry after open poll.
43	10	–	46	10	13
55	6	43	67	7	10
33	0	32	75	14	16
28	2	–	103	7	10
1	0	2	49	0	3
53	0	–	60	19	18
–	–	29	46	10	19
29	0	29	80	15	20
14	0	18	56	3	4
–	–	–	76	16	16
28	2	28	66	7	11
–	–	30	70	20	14
53	25	45	69	14	26
10	0	6	55	1	3
42	2	38	80	8	19
33	0	48	45	9	16
–	–	36	67	2	11
38	9	48	49	2	15
–	–	45	78	22	42
58	0	–	46	2	2

triploids *R. nitidus* and *R. thyrsanthus* showed the lowest stainability, the pentaploid *R. bellardii* resembled the tetraploids and the pentaploid *R. wahlbergii* of the *Corylifolius* group ranked among the highest.

Species that occurred in both Helsingborg and Lund showed significantly correlated values (Tab. 2). Also the Helsingborg values for 1983 were well correlated with those for 1984. This indicates genetic differences between the species as regards pollen viability, and also that these differences are fairly stable between populations from different localities and from season to season. The meiotic irregularities, reported by GUSTAFSSON (1943) and others, are probably the main reason for the low pollen stainability. It should also be noted that almost all unstained pollen grains were much smaller than those that stained and were often considerably deformed.

The TTC values are close to the cotton blue values. Obviously almost all morphologically good pollen grains are also enzymatically active. From the analyses of variance (Tab. 7) it appears, however, as if the TTC test is somewhat more effective for separating species. Both the F-values

Table 2. Spearman rank correlation coefficients for average percentage good pollen for some blackberry species after 3 tests, and after cotton blue tests on material from 2 localities and from 2 seasons

	r_s-value	df	p-value
Helsingborg 1984			
Cotton blue × TTC	0.965	15	< 0.01
Cotton blue × germination	0.405	15	0.07
TTC × germination	0.408	15	0.07
Cotton blue tests			
Helsingborg 1984 × Lund 1984	0.752	11	< 0.01
Helsingborg 1983 × Lund 1984	0.920	15	< 0.01
Helsingborg 1983 × 1984	0.742	15	< 0.01

Table 3. Cotton blue staining in the Helsingborg material, 1983. Means and standard deviations (in italics) are given for each collection date

Species	24/6		29/6		5/7		11/7		14/7	
R. nessensis	43	*6.6*	40	*7.3*						
R. scissus	25	*5.8*	42	*8.8*						
R. plicatus			32	*7.7*	35	*9.5*				
R. sulcatus	33	*7.2*	33	*6.5*						
R. nitidus							3	*1.8*	8	*3.2*
R. affinis							57	*5.4*	39	*19.8*
R. axillaris							26	*13.2*	16	*4.4*
R. insularis					21	*3.5*	34	*6.1*		
R. lindebergii							9	*5.5*	19	*5.3*
R. laciniatus							26	*9.6*	21	*8.4*
R. sprengelii							36	*8.7*	12	*3.1*
R. pyramidalis							27	*7.8*	22	*10.5*
R. polyanthemus							56	*7.8*	26	*15.0*
R. thyrsanthus							10	*5.3*	12	*5.1*
R. bellardii			49	*3.9*	37	*12.4*				
R. hartmannii							59	*10.5*	41	*11.2*
R. radula							32	*10.7*	28	*7.8*
R. taeniarum							42	*10.4*	39	*12.8*
R. vestitus							49	*5.2*	30	*10.3*
R. wahlbergii					52	*8.9*	52	*7.5*		

and the number of significantly different species pairs are higher for TTC. Similarly, the standard deviations for the set of 10 flowers from each species are lower for TTC than for cotton blue (Tabs. 4 and 5).

TTC may be more easily influenced by environmental factors; in the two-way analysis of variance the time factor is more significant in the TTC test than in the cotton blue test (Tab. 7).

Table 4. Cotton blue staining in the Helsingborg material, 1984. Means and standard deviations (in italics) are given for each collection date

Species	20/6		25/6		30/6		4/7		9/7		12/7		16/7	
R. nessensis	47	*9.2*	48	*6.2*										
R. scissus	60	*8.6*	51	*3.9*										
R. plicatus					30	*4.4*	36	*6.7*	35	*5.8*				
R. sulcatus	36	*6.9*	28	*4.7*	30	*7.6*								
R. nitidus											4	*1.5*	2	*2.0*
R. affinis					52	*3.4*	51	*14.5*	50	*7.0*	52	*5.1*	60	*8.5*
R. insularis											27	*5.8*	24	*6.4*
R. lindebergii											22	*8.4*	21	*5.6*
R. sprengelii											31	*6.8*	34	*9.9*
R. polyanthemus											50	*3.7*	62	*7.7*
R. thyrsanthus											10	*2.8*	14	*3.9*
R. bellardii									39	*5.2*	41	*8.6*		
R. hartmannii													31	*7.0*
R. taeniarum											36	*6.9*	40	*8.1*
R. wahlbergii							52	*6.1*	54	*4.5*	57	*4.4*		

Table 5. TTC staining in the Helsingborg material, 1984. Means and standard deviations (in italics) are given for each collection date

Species	20/6		25/6		30/6		4/7		9/7		12/7		16/7	
R. nessensis	41	*6.9*	45	*6.4*										
R. scissus	56	*6.0*	54	*5.5*										
R. plicatus					26	*4.2*	33	*4.6*	41	*5.1*				
R. sulcatus	26	*4.8*	30	*4.4*	28	*3.8*								
R. nitidus											1	*1.5*	2	*1.9*
R. affinis					59	*3.4*	51	*4.4*	51	*4.5*	48	*3.8*	56	*5.3*
R. insularis											29	*3.9*	28	*7.1*
R. lindebergii											14	*7.5*	14	*3.9*
R. sprengelii											27	*3.7*	28	*4.8*
R. polyanthemus											49	*5.1*	57	*4.3*
R. thyrsanthus											8	*2.5*	12	*2.3*
R. bellardii									37	*5.3*	46	*3.7*		
R. hartmannii													33	*5.6*
R. taeniarum											35	*5.2*	44	*5.6*
R. wahlbergii							58	*6.6*	58	*5.3*	57	*4.0*		

In 1984 no time-dependent tendency of increasing or decreasing values for TTC or cotton blue could be found (Tabs. 4 and 5). In 1983 the pollen last collected shows a decrease and also increased standard deviations (Tab. 3). The reason for this is not known but probably has some connection with the weather prevailing at the time of pollen development.

When compared with the data for relative seed set, the cotton blue and TTC values show similar correlations, the cotton blue values being

somewhat better (Tab. 6). In sum, there seems to be a definite association between relative seed set and the occurrence of good pollen.

The germination tests gave consistently lower values than the other tests. This agrees with HARDY (1932); in different raspberry cultivars he obtained 10–95% morphologically good pollen only 25–80% of which germinated.

In the Helsingborg material germination values are even lower than Hardy's. *R. affinis* and *R. wahlbergii,* for example, both produced over 50% morphologically good and enzymatically active pollen. However,

Table 6. Spearman rank correlation coefficients for average percentage good pollen and relative seed set for blackberry species grown in Helsingborg

	r_s-value	df	p-value
Cotton blue 1983 × self-pollination seed set	0.214	20	0.18
Cotton blue 1983 × open pollination seed set	0.383	20	0.05
Cotton blue 1984 × self-pollination seed set	0.488	15	0.03
Cotton blue 1984 × open pollination seed set	0.405	15	0.07
TTC × self-pollination seed set	0.457	15	0.04
TTC × open pollination seed set	0.354	15	0.10
Germination × self-pollination seed set	0.218	15	0.22
Germination × open pollination seed set	0.440	15	0.05

almost none of the pollen germinated. In *R. polyanthemus,* with similar values for cotton blue and TTC, pollen germination was on the other hand fairly high.

CHOMISURY (1927) obtained the very high value of 74% pollen germination for the tetraploid blackberry cultivar Lawton, which was, however, reported to have normal pollen meiosis, resulting in only well-formed grains of even size. These characteristics are often found in *Rubus* hybrids (LIDFORSS 1905, GUSTAFSSON 1943) but are not to be expected in the wild species I have investigated.

As my germination data do not merit parametric treatment, the parameteric analyses of variance should be regarded with caution. Still, one may safely conclude that pollen germination is inferior to the other tests for separating species. Also the highly significant time factor in the two-way analysis points to strong environmental influence. Still correlation between the germination data and relative seed set after open pollination is almost significant.

REDALEN (1976) obtained 58–84% pollen germination with raspberry cultivars. The position of the flowers in the inflorescence or on the flowering cane had no effect on pollen germination. Nor was there any time-dependent trend in germination values.

Table 7. Parametric analyses of variance (Model 1) (F-values) on percentage good pollen investigated by 3 tests, and Kruskall-Wallis analysis of variance (χ^2-values) on percentage good pollen with the germination test. In connection with the parametric analyses the number of significantly different species pairs was determined by Scheffe's a posteriori test

	F-value	df	p-value	%-different pairs	χ^2-value	df	p-value
One-way analysis 15 species, pollen collected on a total of 7 occasions							
Cotton blue	119.0	14/324	< 0.000	67			
TTC	217.6	14/323	< 0.000	76			
Germination	58.5	14/326	< 0.000	37	127.1	14	< 0.000
One-way analysis 8 species, pollen collected on same 2 occasions							
Cotton blue	162.1	7/146	< 0.000	86			
TTC	285.3	7/147	< 0.000	89			
Germination	83.7	7/146	< 0.000	46	113.7	7	< 0.000
Two-way analysis 8 species, pollen collected on same 2 occasions							
Cotton blue							
Time	4.80	1	0.030				
Species	186.7	7	< 0.000				
Interaction	3.14	7	0.004				
TTC							
Time	8.13	1	0.005				
Species	361.6	7	< 0.000				
Interaction	3.90	7	0.001				
Germination							
Time	22.01	1	< 0.000				
Species	121.6	7	< 0.000				
Interaction	7.39	7	< 0.000				

In similar experiments REDALEN (1977) noted relative seed set of 47 to 100% for different raspberry cultivars. These values were, however, not correlated with the corresponding pollen germination values of 78–88%. Crossing experiments carried out on this material showed that maternal

influence was more important than paternal influence for seed set. DAUBENY (1971) in five series of observations of relative seed set and pollen stainability with acetocarmine on raspberry cultivars, obtained a positive correlation between seed set and pollen stainability for only one series. Since raspberries are diploid and amphimictic, pollen meiosis is probably sufficiently regular for pollen quality not to limit seed set.

To sum up, TTC seems to be the best method for distinguishing the blackberry species investigated here. Cotton blue is almost as good, perhaps even somewhat better in correlation tests with relative fruit set. Moreover, it is cheaper and easier to work with. Pollen germination is clearly inferior.

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