Effect of hormones on wheat–barley crosses, embryo rescue and mitotic and isozymic studies in hybrids

Vijay K. Khanna*, S. Dhaubhadel†, S. Kodali* and G. K. Garg†

*Department of Plant Breeding, G. B Pant Agrivarsity, Pantnagar 263 145, India
†Department of Molecular Biology and Genetic Engineering, G. B. Pant Agrivarsity, Pantnagar 263 145, India

The objective of this study was to investigate the effect of application of gibberellic acid and kinetin on pollen germination, pollen tube growth and seed set in wheat–barley crosses and to find out the best timing of their application on flowers in order to get maximum seed set. Embryo rescue was also tried to obtain the hybrid plants, and mitosis and isozymic analysis was done to confirm their hybridity. Seed set was more in barley–wheat cross than in its reciprocal cross. The different hormonal treatments had a drastic effect on pollen tube growth and abnormal pollen tubes. The correlation between pollen tube growth and seed set was also found to be highly significant. Maximum seed was obtained in the crosses where gibberellic acid was applied 5 min after pollination, followed by another application after 24 h. The best age of the hybrid embryos to be rescued from *in vivo* condition was 17–19 days after pollination, which produced healthy-looking plantlets. Out of the five different modified Murashige–Skoog (MS) media with growth regulators, modified MS media with 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.5 mg/l) gave the best results. Mitotic abnormalities of chromosomes in the hybrid root tips were in the form of bridges at anaphase, and somatic chromosome numbers in these were 21 and 28 in most of the cases but in one cell 35 chromosomes could be counted. In both the isoenzymes studied, i.e. peroxidase and esterase, the hybrids showed a combination of both the parental bands, but some new bands also appeared at different positions with different intensities. Some parental bands were missing in all the hybrids.

THE fertilization of wheat with barley appears to be restricted to a very few cultivars[1]. Despite intense efforts since 1973, introgressions of barley traits to bread wheat through wide hybridization appear unattainable due to crossability (Kr alleles) barriers on the homoeologous Group 5 chromosomes of *T. aestivum*[2] and to the apparent lack of pairing between wheat and barley chromosomes[3–5]. A number of workers have attempted to overcome this incompatibility barrier between wheat and barley by the post-pollination application of growth regulators[6–8]. The response of pollen to exogenous growth hormones is not well understood. Plant hormones have been implicated in the growth of pollen tube and fruit set. Many workers have reported the application of hormones 24 h after pollination in wheat–barley crosses[8–10] to improve seed set, but Neeraj and Khanna[11] reported that fertilization takes place within 24 h of pollination.

Embryo culture, though not always essential[12], usually facilitates hybrid production greatly[13] and has been used in many new crosses involving *Hordeum*, *Triticum* and *Secale*[14].

The present experiments were designed to standardize the best timing for the application of the hormones on flowers in order to obtain maximum seed set in wheat–barley crosses, to rescue the hybrid embryos to get plants and to confirm the hybridity of these plants by mitotic studies and peroxidase and esterase isoenzymic patterns.

Materials and methods

Effect of hormones on pollen tube studies and seed set

The experimental material comprised one variety each of *Triticum aestivum* and *Hordeum vulgare*, i.e. UP2121 and Jagrati, respectively. The seeds were sown in the field on the 24th of Nov. and 5th of Dec., 1990. Crosses were made reciprocally in February and March 1991. Two different hormones, namely, gibberellic acid (GA_3) and kinetin (KIN) at a concentration of 75 ppm each were sprayed on the flowers, separately and in combination, at different timings before and after pollination.

The styles along with the stigmas were detached at 120 min after hand pollination and fixed in 1:2 lactoalcohol for 48 h and then preserved in 70% alcohol. Five styles were chosen at random from each spike and all the pollen grains on the stigma were observed for pollen germination. For pollen tube growth, the lengths of the three longest pollen tubes in each style were

Table 1. Pollen germination, pollen tube growth and abnormal pollen tubes after 120 min of pollination and seed set in UP2121 × Jagrati cross

Treatment	Percentage pollen germination	Pollen tube growth (μm)	Abnormal pollen tubes (%)	Seed set (%)	
Control	83 8 ± 11 6	6 2 ± 0.1	4 5 ± 0 2	No hormone	0.56
				GA_3 after 24 h	2 20
				KIN after 24 h	2 00
				GA_3 + KIN after 24 h	0 00
GA_3 application before pollination	71 7 ± 7.8	4 4 ± 0.1	7 5 ± 0 8	No further hormone	0 00
				GA_3 after 24 h	0 00
KIN application before pollination	81 7 ± 6 6	4 5 ± 0 6	7 5 ± 1 4	No further hormone	0 00
				KIN after 24 h	0 00
GA_3 + KIN application before pollination	79 5 ± 11 2	4 4 ± 0 1	7 7 ± 1.1	No further hormone	0 00
				GA_3 + KIN after 24 h	0 00
GA_3 application 5 min after pollination	82.9 ± 10 5	7.9 ± 0.1	2 5 ± 0 3	No further hormone	0 00
				GA_3 after 24 h	2 50
KIN application 5 min after pollination	73 9 ± 10.5	7.7 ± 0.1	3 8 ± 0 9	No further hormone	0 00
				KIN after 24 h	2.22
GA_3 + KIN application 5 min after pollination	77 5 ± 5 4	7 5 ± 0 1	4 5 ± 0 9	No further hormone	0 00
				GA_3 + KIN after 24 h	1 67
GA_3 application 45 min after pollination	89 7 ± 6 6	5 5 ± 0.1	4 0 ± 0.3	No further hormone	1 72
				GA_3 after 24 h	0 00
KIN application 45 min after pollination	76 7 ± 8 1	5.4 ± 0 2	5 6 ± 0 5	No further hormone	0 00
				KIN after 24 h	0 00
GA_3 + KIN application 45 min after pollination	84.3 ± 9 4	5 6 ± 0 1	3 4 ± 0 6	No further hormone	0 00
				GA_3 + KIN after 24 h	1 23
GA_3 application 90 min after pollination	71 5 ± 16 7	5.5 ± 0.4	3 3 ± 0.8	No further hormone	0 00
				GA_3 after 24 h	0.00
KIN application 90 min after pollination	83 8 ± 13.0	5 1 ± 0 0	3.2 ± 0 6	No further hormone	1 82
				KIN after 24 h	0.00
GA_3 + KIN application 90 min after pollination	83 9 ± 2.9	5 4 ± 0 0	7.4 ± 1 0	No further hormone	0 00
				GA_3 + KIN after 24 h	0 00

recorded. These pistils were washed in distilled water and stained with cotton blue solution[15]. The pollen grains and the pollen tubes were stained deep blue, whereas the stylar tissue was either colourless or very lightly stained. The data on pollen germination, pollen tube growth and abnormal pollen tubes were recorded. Some pollinated spikelets were left on the plants to mature in order to calculate the percentage seed set.

Embryo rescue, mitotic and isoenzymic studies of the hybrids

Along with the two varieties used for pollen tube studies, UP2003 variety of wheat and Karan 741 variety of barley were also included for these studies. After 24 h of pollination, 75 ppm GA_3 was sprayed on the florets. This was repeated every 24 h for 4 days. After 13–22 days of pollination, a part of the pollinated panicles was detached from the plants and brought to the laboratory for embryo rescue studies, and the rest was left for seed set. Sterilized seeds were dissected in laminar flow and the embryos were cultured on a modified Murashige–Skoog[16] (MS) medium supplemented with various concentrations and combinations of growth regulators and amino acids. These growth regulators were: 1 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 1 mg/l of indole acetic acid (IAA) and 0.5 mg/l of napthalene acetic acid (NAA). Also added was 0.5 mg/l of KIN (6-furfuryl-amino purine). The amino acids used were: 100 mg/l glutamine, 25 mg/l serine and 500 mg/l casein hydrolysate (CH). The *in vitro* cultured plantlets after development of roots and shoots were transferred to soil and vermiculite (1:1) mixture.

To make slides, root tips were squashed in 1.5% propionocarmine after hydrolysing in 1 N HCl at 60°C for 5–10 min. The data on various chromosomal abnormalities, such as laggards and bridges at metaphase and anaphase stages, were recorded for the study of isoenzymes of peroxidases and esterases. The leaves were homogenized in a pre-chilled mortar and pestle with 3–5 ml of ice-cold 0.9% NaCl solution and 0.2–0.6 g glass beads. The homogenate was stored at 4°C for 6–12 h and then centrifuged at 12,000 × *g* for 45 min in a refrigerated centrifuge. The clear supernatant was carefully decanted and the pellet containing cell debris was discarded. The supernatants were separately filled in dialysis bags and dialysed against 60% sucrose solution at 4°C. Protein estimation was done by UV absorption method.

Table 2. Pollen germination, pollen tube growth and abnormal pollen tubes after 120 min of pollination and seed set in Jagrati × UP2121

Treatment	Percentage pollen germination	Pollen tube growth (μm)	Abnormal pollen tubes (%)	Seed set (%)	
Control	86.5 ± 8.1	6.1 ± 0.1	5.4 ± 0.9	No hormone	1.60
				GA_3 after 24 h	9.38
				KIN after 24 h	6.25
				GA_3 + KIN after 24 h	2.60
GA_3 application before pollination	72.4 ± 12.7	4.6 ± 0.1	4.3 ± 0.7	No further hormone	0.00
				GA_3 after 24 h	4.76
KIN application before pollination	71.1 ± 15.2	4.2 ± 0.1	6.8 ± 1.1	No further hormone	2 52
				KIN after 24 h	0.00
GA_3 + KIN application before pollination	74.9 ± 18.2	4.3 ± 0.1	7.8 ± 1.1	No further hormone	0.00
				GA_3 + KIN after 24 h	0.00
GA_3 application 5 min after pollination	81.3 ± 1.7	7.6 ± 0.1	2.8 ± 0.2	No further hormone	1.80
				GA_3 after 24 h	11.86
KIN application 5 min after pollination	81.7 ± 3.0	7.4 ± 0.3	4.4 ± 0.6	No further hormone	6.67
				KIN after 24 h	8.62
GA_3 + KIN application 5 min after pollination	82.9 ± 7.0	7.3 ± 0.2	6.4 ± 0.8	No further hormone	0 00
				GA_3 + KIN after 24 h	2.50
GA_3 application 45 min after pollination	74.1 ± 18 7	5.2 ± 0.1	3.8 ± 0.6	No further hormone	9.52
				GA_3 after 24 h	0 00
KIN application 45 min after pollination	74.8 ± 12.1	5.4 ± 0.2	5.6 ± 0.5	No further hormone	0 00
				KIN after 24 h	6.45
GA_3 + KIN application 45 min after pollination	87.2 ± 2.4	5.1 ± 0.1	3.6 ± 0.6	No further hormone	1.27
				GA_3 + KIN after 24 h	11.67
GA_3 application 90 min after pollination	83.2 ± 3.6	5.3 ± 0.1	3.8 ± 0.5	No further hormone	0.00
				GA_3 after 24 h	10.71
KIN application 90 min after pollination	60.9 ± 15.5	5.2 ± 0.1	6.0 + 0.5	No further hormone	0.00
				KIN after 24 h	5.00
GA_3 + KIN application 90 min after pollination	79.3 ± 11.6	5.1 ± 0.2	6.7 ± 0.9	No further hormone	1 60
				GA_3 + KIN after 24 h	3.33

Table 3. Correlation between pollen tube development and seeds set in UP2121 × Jagrati

Parameter	Correlation coefficient
Percentage pollen germination	+ 0.184
Percentage abnormal pollen tubes	− 0.762**
Pollen tube growth (μm)	+ 0.812**

**Highly significant.

Table 4. Correlation between pollen tube development and seeds set in Jagrati × UP2121

Parameter	Correlation coefficient
Percentage pollen germination	+ 0.522
Percentage abnormal pollen tubes	− 0.670"
Pollen tube growth (μm)	+ 0.590'

'Significant.

"Highly significant.

For polyacrylamide gel electrophoresis the combined methods of Laemmli[17], Blackshear[18] and Garfin[19] were followed. Peroxidase isoenzymes were detected by the method of Liu[20] and esterase isoenzymes by the method of Brewbaker *et al.*[21]. Depending upon their electrophoretic mobility, the observed isoenzymes of peroxidase and esterase were divided into four and five groups, respectively. The bands in these groups have been represented by subscripts a, b, c, ... ,r, as was the case, in increasing order of the electrophoretic mobility within the group.

Results and discussion

Pollen tube studies and seed set

The different hormonal treatments had no significant effect on percentage pollen germination in UP2121–Jagrati cross or its reciprocal cross (Tables 1 and 2). According to Linskens and Kroh[22], plant growth substances have variable, although usually inhibitory, effects on pollen germination in different species. Effects are primarily dependent on hormone concentration, but there may be interactions with other hormones[23].

There was a drastic effect of different hormonal treatments on pollen tube growth in both the crosses. When GA_3 was applied 5 min after pollination, pollen tube growth was good and the seed set was the

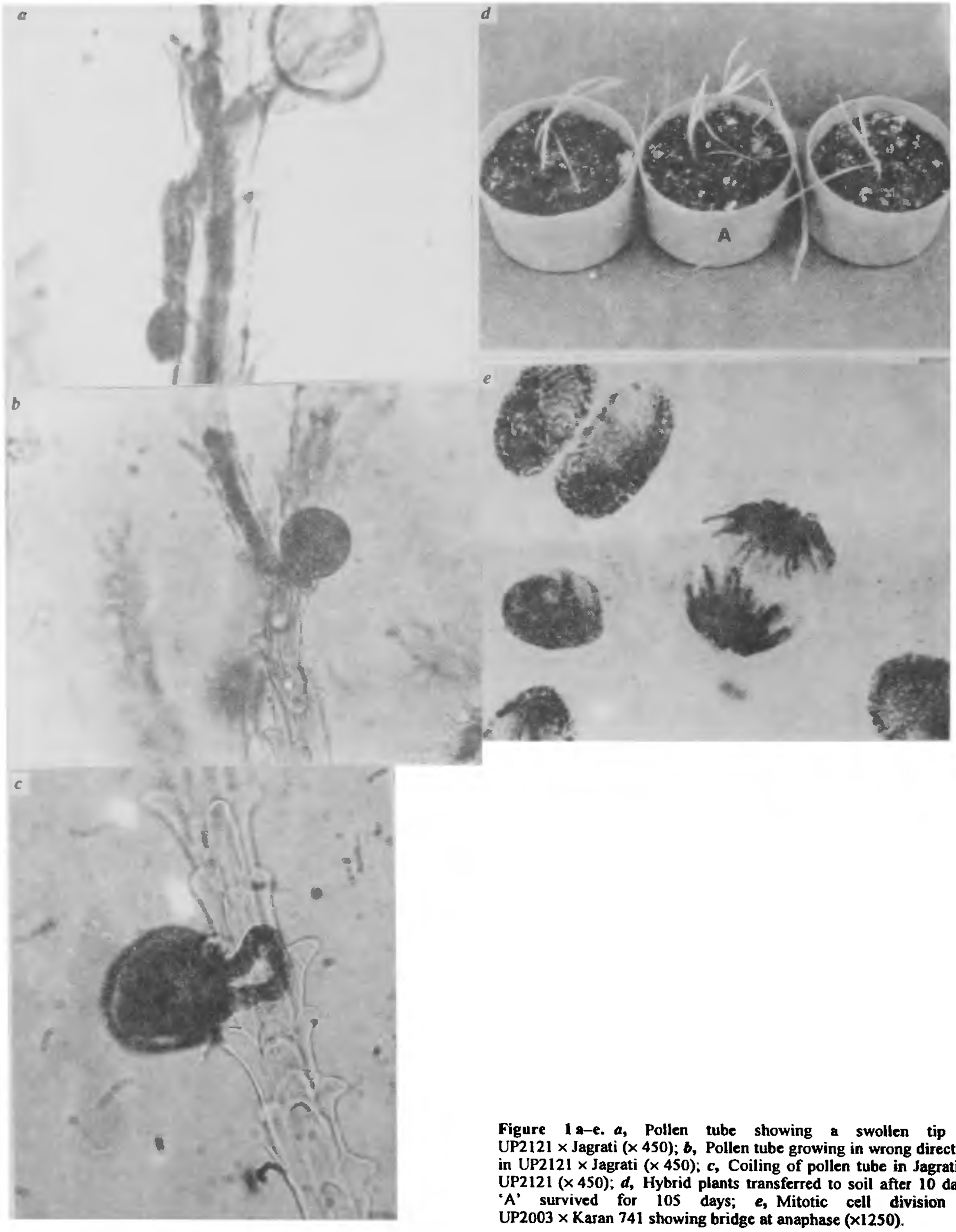

Figure 1 a–e. *a*, Pollen tube showing a swollen tip in UP2121 × Jagrati (× 450); *b*, Pollen tube growing in wrong direction in UP2121 × Jagrati (× 450); *c*, Coiling of pollen tube in Jagrati × UP2121 (× 450); *d*, Hybrid plants transferred to soil after 10 days. 'A' survived for 105 days; *e*, Mitotic cell division in UP2003 × Karan 741 showing bridge at anaphase (×1250).

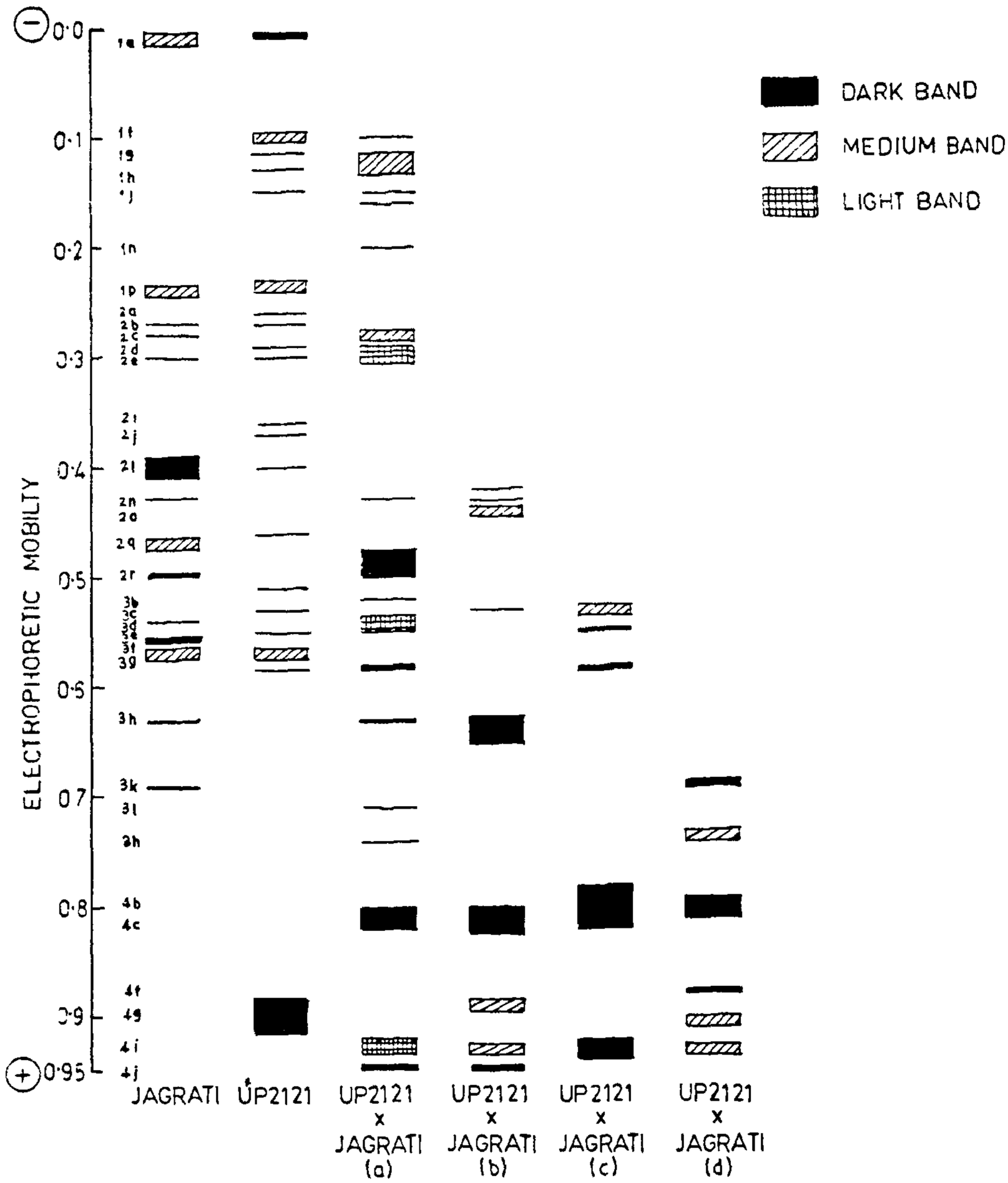

Figure 2. Schematic representation of zymograms showing the electrophoretic pattern of leaf peroxidase in Jagrati, UP2121 and four different UP2121 × Jagrati hybrids (a–d).

Table 5. Percentage seed set in different intergeneric crosses between wheat and barley

Crosses	Number of florets pollinated	Number of seeds formed	Seed set (%)
Self			
UP2003 × UP2003	100	89	89
UP2121 × UP2121	100	88	88
Jagrati × Jagrati	100	89	89
Karan 741 × Karan 741	100	91	91
Wheat × barley			
UP2003 × Jagrati	3140	86	2.73
UP2003 × Karan 741	440	9	2.05
UP2121 × Jagrati	1780	9	0.51
UP2121 × Karan 741	500	7	1 40
Barley × wheat			
Jagrati × UP2003	1400	114	8.14
Jagrati × UP2121	1732	54	3 11
Karan 741 × UP2003	520	23	4 42
Karan 741 × UP2121	410	12	2.90

maximum (Tables 1 and 2). Bose[24] in *Pisum sativum* and Fahnrich and Ullrich[25] in *Petunia hybrida* also found that GA_3 stimulated pollen tube growth. Shukla and Tewari[23] noticed that, although IAA, GA_3 and KIN can also stimulate pollen tube growth in *Calotropis procera*, when anyone of these is combined with ABA, tube growth falls below that of control. According to them, the effects are primarily dependent on hormone concentrations, but there may be interactions with other hormones.

There was a significant correlation between pollen tube growth and seed set (Tables 3 and 4). By comparing the pollen tube development with the time needed for the first tube to reach the embryo sac, Hoshikawa[26] observed in wheat that only the early germinating grains and the fast growing pollen tubes are of interest with regard to fertilization.

A study was made on the development of pollen tubes in the different crosses to find out whether the checking of normal growth of pollen tubes in some cases was due

Table 6. Effect of various media on the growth and development of hybrid embryos

Media	Number of embryos cultured	Number of embryos showing growth	Embryos showing growth (%)	Growth response
MSM + 2,4-D	40	27	67 50	+ + +
MSM + 2,4-D + KIN	207	166	81.16	+ + + +
MSM + IAA + KIN	69	32	46 37	+ +
MSM + NAA + KIN	56	21	37.50	+ + + +
MSM + CH + IAA + KIN	55	42	76 36	+ + +

+ + Most of the embryos stopped growing after 3 weeks, plantlets thin and weak.
+ + + Callusing with limited plantlet growth.
+ + + + Healthy plants/frequent formation of multiple shoots and roots.

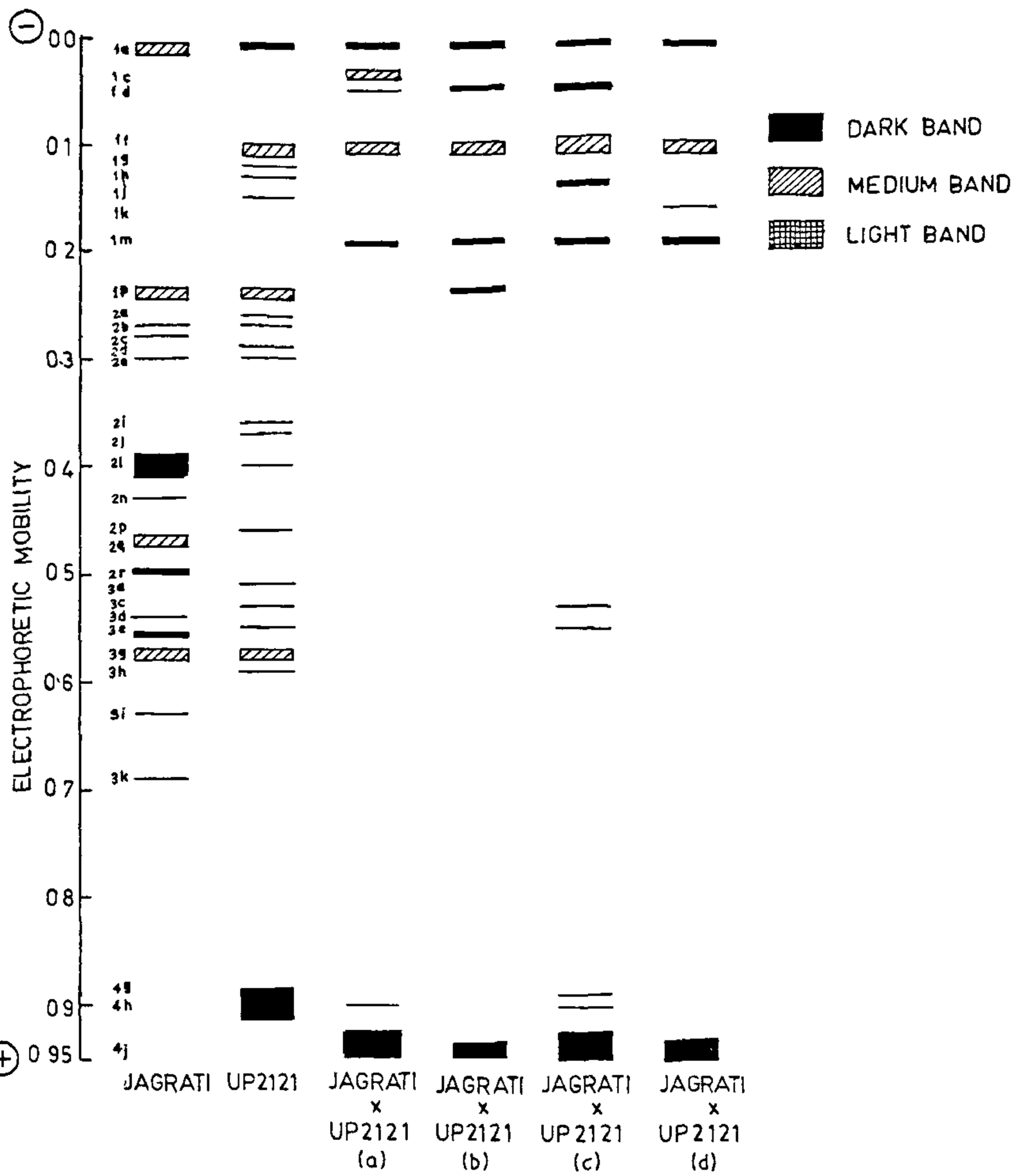

Figure 3. Schematic representation of zymograms showing the electrophoretic pattern of leaf peroxidase in Jagrati, UP2121 and four different Jagrati × UP2121 hybrids (a–d)

to their abnormal development. A striking aberration was the occurrence of the swelling of the pollen tube tip, which had the appearance of being filled with a dense cytoplasm (Figure 1*a*). Many pistils showed no swellings at all, others had one, two or several pollen tubes with swollen tips. Other aberrations like pollen tubes growing in the wrong direction (Figure 1*b*) or bursting of the tip of the pollen tube also occurred. Some showed

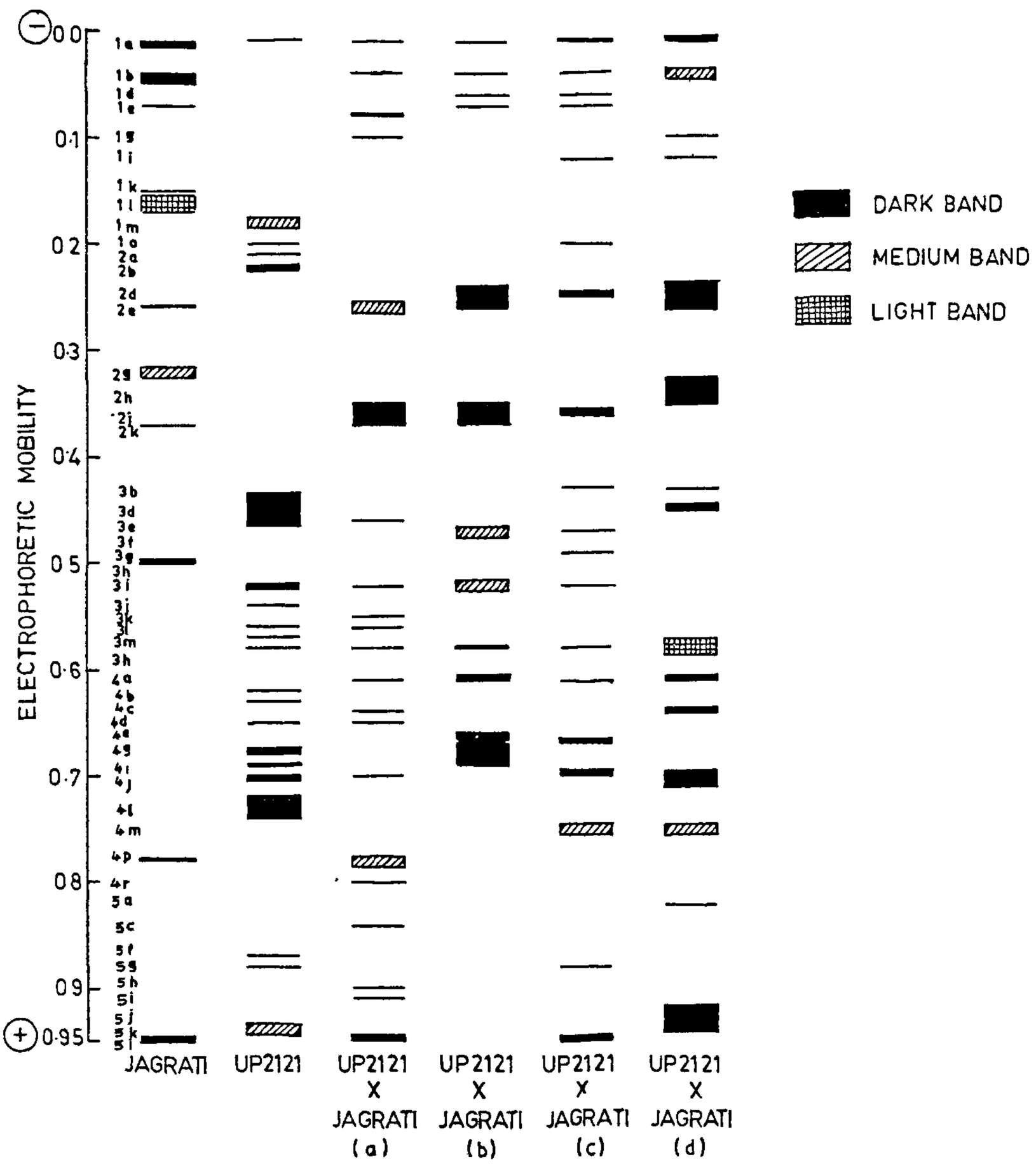

Figure 4. Schematic representation of zymograms showing the electrophoretic pattern of leaf esterase in Jagrati, UP2121 and four different UP2121 × Jagrati hybrids (a–d).

Table 7. Cytological abnormalities (bridges) at anaphase during mitosis in different wheat–barley crosses

Crosses	Total cells studied	Cells with bridges	% cells with bridges
UP2003 × UP2003	100	–	–
UP2121 × UP2121	100	–	–
Jagrati × Jagrati	100	–	–
Karan 741 × Karan 741	100	–	–
UP2003 × Jagrati	133	3	4.51
UP2003 × Karan 741	123	12	4.75
UP2121 × Jagrati	164	15	4.14
UP2121 × Karan 741	135	2	1.48
Jagrati × UP2003	166	12	7.22
Jagrati × UP2121	112	20	17.85
Karan 741 × UP2003	106	4	3.77
Karan 741 × UP2121	118	6	5.08

twisting or coiling (Figure 1*c*) of the pollen tube in the stigmatic hairs. In wheat–barley crosses there was an increase in the pollen tube abnormalities when GA_3 or GA_3–KIN were applied before pollination. In all those treatments where abnormalities were high there was no seed set. In the reciprocal cross also seed set was nil or less when abnormalities were more, whereas when these were low seed set was better.

Presence of abnormal pollen tubes have been reported in other crops also. Singh[27] observed that incompatible pollen tubes in rape and mustard were short and often twisted. These did not penetrate the stigmatic tissue. Stott[28] reported that in apple cultivars incompatible tubes grew slowly, often stopped completely and had a heavy deposition of callose tissue along and at the end of the tube. Khanna and Chowdhury[29] recorded pollen tube abnormalities in the form of swelling at the tip or its bursting on selfing *Brassica chinensis* or *B. juncea*.

Keeping all the observations in mind it may be concluded that when wheat-barley crosses were made, crossability seemed to be poor but it was better in the

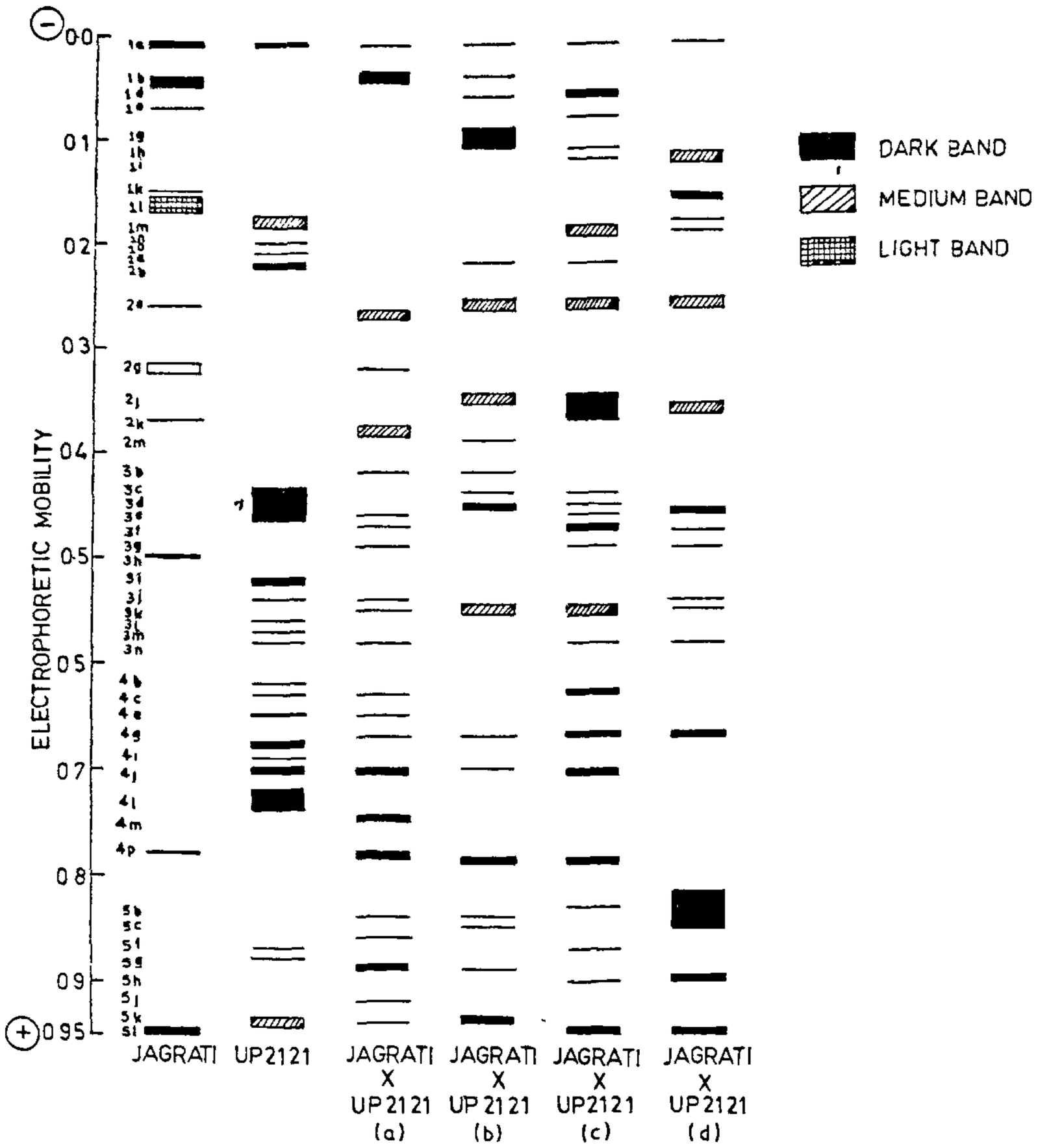

Figure 5. Schematic representation of zymograms showing the electrophoretic pattern of leaf esterase in Jagrati, UP2121 and four different Jagrati × UP2121 hybrids (a–d)

reciprocal crosses. Poor seed set could be on account of slow pollen tube growth and a higher percentage of abnormalities. Seed set improved after application of hormones 5 min after pollination, followed by another application after 24 h. Since fertilization in wheat–barley crosses takes place within 24 h of pollination[11], hormones play some role at pre- as well as post-fertilization stages to improve seed set.

Seed set was found to be more in case of selfings (Table 5). It was more when barley was taken as the female parent and wheat as the male parent compared to the reciprocal cross. According to Fedak[3] and Islam *et al.*[30], a wheat–barley cross is more difficult than a barley–wheat cross. Neeraj and Khanna[11] reported 6.7% seed set in *Triticum aestivum* × *Hordeum vulgare*, whereas Kodali[31] obtained only 0.56% seed in the same cross, though in the reciprocal cross she reported 1.6%.

Embryo rescue studies

The success of embryo rescue is influenced by the age of the embryo at the time of its culture. The best age of the embryo was found to be 17–19 days after pollination (DAP), for which the plantlets produced were healthy and showing growth. Embryos younger than this, i.e. 13–16 DAP, gave lean and weak plantlets and in some cases sparse callusing was also observed. The embryos cultured 20–22 DAP did not give better results as the abortion process had already started.

Out of the five different concentrations and combinations of growth regulators with modified MS media, the one with 1 mg/l 2, 4-D and 0.5 mg/l KIN proved to be the best, giving 81.2% embryo growth (Table 6). A critical consideration in embryo culture is the composition of the culture media[32, 33]. Monnier[34] felt that hormonal supplements were not necessary for embryo

culture as embryos possess an endogenous hormone supply. Nevertheless, some investigators feel that hormones may have a profound influence on embryo development[35, 36].

Plantlets provided with Hoagland's solution supported good growth compared to those kept in the liquid MS medium supplemented with growth regulators. Plantlets transferred directly from the test tubes to the potted soil did not survive at all. Out of the 477 plantlets obtained, 60 were transferred to the soil but only one plant could survive for 3 months and 12 days, with the growth of the plant being abnormal and at a slower pace. Most of the other plantlets survived for 7–10 days (Figure 1*d*).

Mitosis of parents and hybrids

Mitotic studies showed that the wheat parents had 42 chromosomes and barley 14. Chromosome number variation was observed in the root tip cells of the plantlets of the hybrids. Some plantlets had 28 chromosomes, as expected, comprising 21 of wheat and 7 of barley. Others had 21 chromosomes, which were assumed to be haploids of wheat. One plantlet had 35 chromosomes, which could be the result of an unreduced pollen with 14 chromosomes from barley fertilizing the egg of wheat having 21 chromosomes. Many cells in the hybrids showed bridges (Figure 1*e* and Table 7). According to Bennett[37] nuclear instability in triticale endosperm often began with the formation of a chromatin bridge at anaphase. In triticale, the replication of rye genome takes longer to complete S-phase than wheat. Under certain circumstances, rye chromosomes may enter mitosis, having failed to complete replication at one or both telomeres. This results in bridge formation at anaphase. Bridge formation in wheat–barley hybrids may be because of similar reasons.

Peroxidase isoenzymes in parents and hybrids

A total of 20 and 13 peroxidase bands were observed in UP2121 and Jagrati, respectively (Figures 2 and 3). In UP2121, six bands were seen in group 1, eight in group 2, five in group 3 and one band in group 4. In Jagrati, two bands were seen in group 1, seven in group 2, four in group 3 and none in group 4.

In the case of Jagrati–UP2121 cross (Figure 3) band number 1a was seen in all the plantlets studied. It was also present in both the parents. The band 1f of UP2121 was seen in all the plantlets but the bands 2b to 3k were absent in all except one (c), where bands 3c and 3e appeared. Some new bands, namely 1m and 4j appeared in all the plantlets.

In UP2121–Jagrati cross (Figure 2) band number 1a was absent in all the plantlets while it was present in both the parents. One of the plantlets showed similarity to UP2121, having UP2121 specific bands, namely 1f, 1g, 1h, 1j and 2d. Some of the Jagrati specific bands, namely 2c, 2n, 2r, 3g, 3h and 3k, were also seen. The peroxidase pattern of the plantlets obtained from this cross showed similarity to UP2121 than to Jagrati, but plantlet (d) showed none of the UP2121 bands.

Esterase isoenzymes in parents and hybrids

In esterases, a total of 21 and 11 bands were observed in UP2121 and Jagrati, respectively (Figures 4 and 5). In UP2121, three bands were present in group 1, two in group 2, six in group 3, seven in group 4 and 3 in group 5. In Jagrati there were 5 bands in group 1, three in group 2 and one band in groups 3, 4 and 5.

In UP2121–Jagrati cross (Figure 4) five bands were UP2121-specific in one of the plantlets (c), while the same plantlet had two Jagrati-specific bands. Jagrati-specific bands 1k, 1l and 3h and UP2121-specific bands 1m, 2a, 2b and 5f were absent in all the plantlets. Many new bands also appeared. Some plantlets resembled more with Jagrati but they also had many bands common with UP2121. In Jagrati–UP2121 cross (Figure 4) the plantlets resembled the wheat parent more than barley. Jagrati-specific bands 2e, 4p and 5l and UP2121-specific bands 3d, 3j, 4j and 5k were present in all the plantlets. Many new bands, e.g. 1g, 1h, 2j, 3f, 5b, 5f, 5h and 5j were also seen. When the crosses were compared with their reciprocals, the progenies obtained differed in their esterase pattern. Fedak[38] studied the esterase pattern of leaf tissue in wheat–barley hybrids. The hybrid pattern was a combination of the differential parental bands.

Eucaryotic cells contain a large set of sequence-specific DNA binding proteins whose main function is to turn genes on or off. Each of these gene regulatory proteins is present in relatively few copies per cell and their binding to DNA either facilitates or inhibits the transcription of other genes. So, the appearance of many new bands or the disappearance of a whole cluster of bands, as seen in our study, may be due to the regulatory protein, the gene responsible for which might have been lost during cell division. The presence of bands with the same electrophoretic mobility but different intensity must be accounted for by the fact that the responsible gene or genes occur at different stages of activities[39]. Isozyme polymorphism within and between species is a result of gene recombination, gene mutation and selection[40].

Along with the information of best timing to apply hormones for better seed set in wheat–barley crosses, the best age of hybrid embryos to be rescued, it was also found as to which was the best modified MS medium, out of the five combinations tried, for plant regeneration. The mitotic and isozymic studies confirmed the hybridity of the plantlets obtained.

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