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Study of pre-fertilization parameters in wheat-maize crosses

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Abstract

The present study aimed at investigation of pollen tubes and stigma or style interactions in terms of different parameters, which were chosen to quantify the success of wheat-maize hybridization. Eighteen F_1s derived from nine cold tolerant and two cold susceptible wheat varieties were crossed with maize (*Zea mays*) genotype 'Pragati'. Mean percent pollen germination and mean pollen tube growth were found to be maximum in Druchamp/UP 2425 Pragati cross (62.48% and 174.66 mm, respectively) with the lowest percentage of abnormal pollen tubes. Embryo Formation Frequency (EFF) showed highly significant and positive correlation with percent pollen germination (0.986) and pollen tube length (0.965). Histological studies indicated aberrant secretions of proteins and carbohydrates in the wheat-maize crosses. It was concluded that the two crosses *viz*. Druchamp with UP 2425, and UP 2590 would provide exciting possibilities for further investigations in haploid production by wheat-maize crosses and QTL mapping for cold tolerance in wheat.

Key words: Wide hybridization, *Triticum aestivum*, *Zea mays*, Fluorescence microscopy

Introduction

Wide hybridization with elimination of genome of one parent has been an attractive method for induction of haploid zygotic embryos and plant formation. Bulbosum technique, originally developed for barley haploid production, was extended to wheat with high frequency of haploid generation [1]. However, the deleterious effect of crossability genes Kr_1 and Kr_2 in wheat has restricted use of the bulbosum technique for poly-haploid production in wheat [2]. The production of haploid plants from inter-generic crosses between wheat × maize was reported by Laurie *et al*. [3]. Since then, it has emerged as the system of choice for poly-haploid production in wheat because in this system fertilization by maize has been found to be comparatively insensitive to action of dominant alleles at Kr loci. Thus, haploids can be recovered across different genotypes. The production of (doubled) haploid plants is advantageous for plant breeders because it offers a way of rapidly advancing chosen lines to complete homozygosity and of increasing the efficiency of subsequent selection process [4, 5]. In many distant hybridizations, although fertilization occurs normally and embryos begin to develop in a relatively normal way, a number of irregularities subsequently set in that result in the eventual death of embryos and collapse of seeds [6]. Embryo rescue, though not always necessary usually facilitates hybrid production greatly. It is now well established that the abortion of embryo in inter-specific and inter-generic crosses is preceded by disintegration of the endosperm, thereby depriving the embryo of source of nutrients. Importance of various carbohydrates, proteins and callose in preventing or permitting pollen tube growth towards the egg for fertilization in wheat x barley [7] and wheat x rye [8] crosses has already been reported.

Since wheat x maize crosses show low frequency of embryo formation and haploid production, it becomes important to know the causes of pre-fertilization barriers such as inability of pollen tubes to go beyond base of the style and into the ovary wall. In this study, we investigated various pre-fertilization factors like pollen germination, percent pollen tube growth and percent abnormal pollen tube development in eighteen different F_1s of wheat that were crossed with maize composite Pragati. These values were compared with embryo formation frequency (EFF) for each cross. Subsequently, a qualitative assessment of normal and abnormal pollen

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tube development was made using light and fluorescent microscopy. Our hypothesis is, if these parameters are identified in wheat-maize crosses, it can help researchers to identify good cross combinations that can give higher haploid embryos yields in a series of cross combinations for a particular trait of interest like drought tolerance, cold tolerance etc. Winter injury is a major economic risk factor in wheat production. Although genetic differences exist among wheat genotypes, it has been difficult to develop more cold-tolerant cultivars because frost tolerance is a complex trait regulated by multiple genes [9]. Therefore, a fine map for cold-tolerant loci can facilitate understanding the barriers involved and identification of more tolerant varieties. This can be accomplished soon by generating a doubled haploid population involving parents having cold tolerance trait. So in this study, we identified the genotypes of cold tolerant wheat which can produce comparatively high number of embryos during wide-hybridization with maize.

Materials and methods

In the present investigation wheat was sown under normal environmental conditions of *rabi* crop season. Simultaneously maize pollen parents were grown in a controlled greenhouse environment at 19-21°C, and 14-16 h light with the intensity of 400-800 $\mu Em^{-2}s^{-1}$ supplied by sodium lamps. Eighteen F_1s derived from crosses between nine cold tolerant (Dorade 5, 90 Zhong 65, Fanjai 2, VL 829, VL 818, Louhe 6210, Druchamp, VL 738, HS 240) and two cold susceptible wheat genotypes (UP 2590, UP 2425) were used for the study.

F_1s used in this study are denoted by Wn and are as follows: W_1 = VL 829 x UP 2425, W_2 = Dorade 5 x UP 2425, W_3 = Dorade 5 x UP 2590, W_4 = Druchamp x UP 2425, W_5 = 90 Zhong 65 x UP 2425, W_6 = 90 Zhong 65 x UP 2590, W_7 = VL 818 x UP 2425, W_8 = VL 738 x UP 2425, W_9 = VL 818 x UP 2590 , W_{10} = Louhe 6210 x UP 2425 , W_{11} = HS 240 x UP 2425, W_{12} = Louhe 6210 x UP 2590, W_{13} = Fanjai 2 x UP 2425, W_{14} = Druchamp x UP 2590, W_{15} = HS 240 x UP 2590, W_{16} = VL 829 x UP 2590, W_{17} = Fanjai 2 x UP 2590, W_{18} = VL 738 x UP 2590.

For making intergeneric crosses, maize composite variety Pragati was taken. Crossing method given by Laurie and Bennett [3] was used with certain modifications.

The pollen tube studies were done by normal and fluorescent microscopy. The crossed spikes were collected at different time intervals after hand pollination and the slides were prepared as per D'Souza [10], with certain modifications as follows:

For normal microscopy

Observations were recorded on three replications. The crossed spikes were collected at 30 min, 2 h, 6 h, 9 h and 24 h after hand pollination and fixed immediately in 1: 3 aceto-alcohol for at least 24 hrs and then preserved in 70 per cent alcohol till further use. For pollen germination and pollen tube growth observations, the spikelets were gently rinsed in distilled water and pistils were separated from the spikelets after which they were kept in a drop of 1N HCl for 10 minutes. These were again rinsed in distilled water and stained in 1 per cent aniline blue. This stain was prepared by dissolving one gram of aniline blue in 100 ml of lactophenol, a 1:1:1:1 solution of lactic acid, phenol, glycerol and water. After staining, the styles were destained for 10 to 30 minutes in 1:1:1 mixture of 40 per cent acetic acid, orthophosphoric acid and distilled water until the extra stain from stylar tissues was removed. The pistils were then placed in a dish of water to wash out the destaining mixture and then mounted in pure lactic acid for observations under the microscope. The pollen grains and pollen tubes stained deep blue while the stylar tissues were lightly stained. The observations and photographic imaging were performed on Olympus VANOX-S instrument.

For fluorescent microscopy

Styles to be observed were fixed in solution containing formalin 1 part : 80 % alcohol 8 part : acetic acid 1 part by volume (FAA) for 24 h or more. After rinsing in tap water, these were treated in a nearly saturated aqueous solution of sodium hydroxide (about 8 N) for 8 to 24 h to clear and soften the tissue and to permit adequate penetration of the dye. The softened styles were then transferred to a small beaker of tap water for one or more hours to remove most of the sodium hydroxide. Staining was accomplished next in a 0.1 per cent solution of water-soluble aniline blue dye in 0.1 % N Na_3HPO_4 (pH = 11). Then pistils were placed on a glass slide in a drop of glycerin and covered by a cover slip. Nikon ECLIPSE/E-1000 with UV-2A filter and DXM-1200I digital camera was used for study and photography.

With the help of these two techniques, data related to different parameters were collected that included: i) Pollen germination, calculated as percentage of $N_g/N_{T,g}$, where N_g is the number of germinated pollen grains and $N_{T,g}$ is the total number of pollen grains on the stigma; ii) Pollen tube growth, where the rate of pollen tube growth was measured with an ocular micrometer and the lengths of only the three largest pollen tubes were recorded; iii) Abnormal pollen tubes calculated as

percentage of $N_a/N_{T,t}$, where N_a is the number of abnormal pollen tubes and $N_{T,t}$ is the total number of pollen tubes; iv) Seed formation and Embryo formation frequency (SFF and EFF) measured as percentage of (a) $N_s/N_{T,f}$, and (b) N_e/N_s respectively, where N_s is the number of seeds obtained, N_e is the number of embryos obtained, and $N_{T,f}$ is the total number of florets pollinated. One-way analysis of variance (ANOVA) and t-test were used to identify statistically significant differences among treatments at $P < 0.01$ significance level. Standard errors of mean of characters were calculated following the method given by Singh *et al.* [11]. Correlation coefficients between different observed characters were estimated following statistics of Panse and Sukhatme [12]. The secretions produced in the cells of the style, the ovule and obturator were studied at 8 and 48 hrs after pollination in the longitudinal sections of the ovary in wheat selfings and wheat × maize crosses. The prepared samples were treated with Coomassie brilliant blue [13] for protein staining and with Periodic acid - Schiff's reagent [14] for carbohydrate staining.

Results and discussion

Pollen germination

As an event of pollen-pistil interaction, the nature of germination of pollen grains on the stigmatic surface following self and cross pollination was studied. The onset of pollen grain germination was found to be variable in selfings and crosses but the majority of pollen grains in both cases germinated between 15-30 min after pollination (Fig. 1A). There were a small proportion of pollen grains that had either delayed germination or did not germinate at all. Mean percent pollen germination was highest in selfing of W_4 (66.26%) and lowest in W_3 (60.35%).

In wheat x maize crosses, at 30 min and 24 h, pollen germination was recorded highest for W_4 x maize (32.31% and 91.48% respectively) and lowest for W_3 x maize (17.49% and 72.09% respectively). Mean percent pollen germination was also recorded maximum in W_4 x maize (62.48%) followed by W_{14} x maize (61.69%) while a minimum was recorded for W_3 x maize (45.25%). The percent pollen germination parameter had a significant and positive correlation with pollen tube length (0.993**) and percent embryos formed (0.986**), while a negative significant correlation was observed with percent abnormal pollen tubes (–0.975**) (Table 1).

According to Gao *et al.* [15], maize pollen needed approximately 30 min to germinate on the wheat stigma whereas the whole progameic phase in wheat selfings took only 25 min. Heslop and Harrison [16] reported in maize that a pollen grain stored for 2 hours that had lost nearly half of its water content took minutes to germinate, while freshly released pollen germinated in 100 seconds. So it is always recommended to collect fresh pollens just before pollination. The number of pollen grains deposited on the stigma is much higher than the number of pollen tubes required for fertilization. Consequently, a small decrease in pollen germination ability would not seem to be directly responsible for the low fertility of wheat genotype.

Pollen tube growth

After some time of coming in contact with the receptive feathery stigma, the pollen grains first germinated into a pollen tube that later grew through the papillated stigma hair into the long style (Fig. 2A). W_4 (Druchamp x UP 2425) selfing and cross with maize consistently recorded the maximum growth at all measured intervals that was closely followed by W_{14} (Druchamp x UP 2590) genotype with a negligible difference between the two genotypes (Fig. 1B). W_3 (Doradae 5 x UP 2590) genotype showed the minimum growth rate at all data points, which was closely followed by W_2 (Doradae 5 x UP 2425) genotype. The mean pollen tube length for selfings was observed to be highest for W_4 (174.66 mm)

Table 1. Correlation studies for various characters in wheat x maize crosses.

Characters	Pollen tube tube length (µm)	Percent abnormal pollen	Percent seed set	Percent embryo formation	Percent haploids obtained
Percent pollen germination	0.993**	–0.975**	0.501*	0.986**	0.825*
Pollen tube length (mm)		–0.966**	0.380*	0.965*	0.729*
Per cent abnormal pollen tubes			–0.462*	–0.982*	–0.819*
Per cent seed set				0.621*	0.810*
Per cent embryo formation					0.912**

*,**Significant at 1 and 5 per cent level of significance, respectively.

and minimum for W_3 genotype (135.80 mm). Likewise, in wheat x maize crosses, the maximum mean pollen tube length was observed for W_4 x Pragati (145.33 mm) while the minimum was observed for W_3 x Pragati (99.03 mm). Further, a positive and significant correlation (0.965*) was observed with percent embryos formed and a negative and significant correlation with percent abnormal pollen tubes (–0.966**) (Table 1).

The results of pollen tube growth are comparable across different genotypes in both selfings and wheat x maize crosses. It was observed that the rate of pollen tube growth in most of the crosses was comparatively faster at 9 h i.e. showing a considerable increase over a period of 3 h (between 6-9 h; compare the relative length of a stack to a previous stack in any bar in Fig. 1B). After 6 h of pollination, more differentiation and elongation of pollen tubes were observed. Therefore, this time period after pollination is a crucial point for pollen growth and fluctuation of different environmental factors like light intensity, temperature etc. during this period can check the embryo formation frequency. Beyond this period, the growth was observed with fluorescent microscopy because the germinating pollen tubes are more clear and visible till the end of the style. A positive significant correlation (0.965*) was observed between pollen tube growth and embryo formation in wheat × maize crosses (Table 1). Thus growth of pollen tube on stigma in a wide cross can be a good indicator of their compatibility though it is not the only factor that shows cross-ability between them. It is clearly evident that wheat F_1s with longest mean pollen tube growth showed the highest frequency of embryo formation and so on. These results are comparable with those of wide crosses of *H. vulgare* [17].

Abnormal pollen tubes

A study was made on abnormal behavior of pollen tube growth in wheat selfings and wheat x maize crosses. Such abnormalities are in the form of coiling of the tubes (Fig. 2B), two pollen tubes coming out of the same pollen grain (Fig. 2C), swelling of pollen tubes (Fig. 2D) or pollen tubes bifurcating (Fig. 2E), or twisting (Fig. 2F). Swelling and coiling of the pollen tubes accounted for the largest number of aberrations. In case of swelling, the pollen tube tip seemed to be filled with a dense cytoplasm. These occurred in the hair as well as in the transmitting tissue of the style. There was a continuous increase in percent abnormal pollen tubes from 30 min to 24 h. The average percent abnormal pollen tube was highest for W_3 selfing (3.14%) and minimum for W_4 selfing (0.92%). The average percent abnormalities were highest for W_2 x Pragati (9.42%) and lowest for W_4 x Pragati (2.69%) (Fig. 1C).

Comparison of proteins and carbohydrates secretion in wheat selfings and wheat-maize crosses

Coomassie blue was used to stain proteins in cells. In the case of selfing of W_3, at 8 h after selfing, a constant and uniform stain was observed in the extra-ovarian tissues including obturator and ovule (Fig. 3A). At 48 h post-pollination, the ovular tissue and obturator took a darker stain than the adjoining extra-ovarian tissues (Fig. 3B). When W_3 was crossed with maize, the ovule and obturator took light and scattered stain as compare to selfing and after 48 h of cross pollination, the endosperm started to degenerate and there was an increase of the empty spaces in the ovule with the passage of time. (Fig. 3D and 3E). Presence of carbohydrate was detected by Periodic acid-Schiff's (PAS) staining. Immediately after selfing of W_3 genotype, carbohydrates were produced in the extra-ovarian region and in the obturator and this sequence was continued upto 48 h of selfing. When wheat W_3 was crossed with maize, at 48 h after pollination, there was complete absence of carbohydrates on the entire extra-ovarian portion along with the obturator and endosperm could be seen to be degenerating (Fig. 3C and F).

In wheat selfings, the percentage of abnormal pollen tubes was very low at 30 min after pollination. In wheat x maize crosses, the abnormalities were more frequent as compared to wheat selfings. The W_2 x Pragati cross recorded the maximum percent abnormal pollen tubes (14.4%) at 24 h after pollination while minimum (3.83%) was observed for W_4 x Pragati cross (Fig. 1C). The occurrence of abnormalities showed a direct negative correlation with percent embryo formation (–0.982*). It is evident that wheat stigma and style creates more resistance towards maize pollen tube. Therefore, it may be beneficial to use chemical substances released during selfings of wheat to cope up the problem of abnormalities in wheat x maize crosses. Our results confirm that in the case of selfing, the pistil and ovule were immediately prepared to permit pollen tube to grow by secreting carbohydrate and protein supplements. When W_3 was crossed with maize, in initial hours after pollination, there was no stain observed in the obturator region, but the extra ovarian tissues were lightly stained, showing absence of secretions those are necessary for pollen tube growth. At 48 h post pollination degeneration of endosperm and embryo occurs in the ovule along with absence of secretion in this cross. These secretions may be

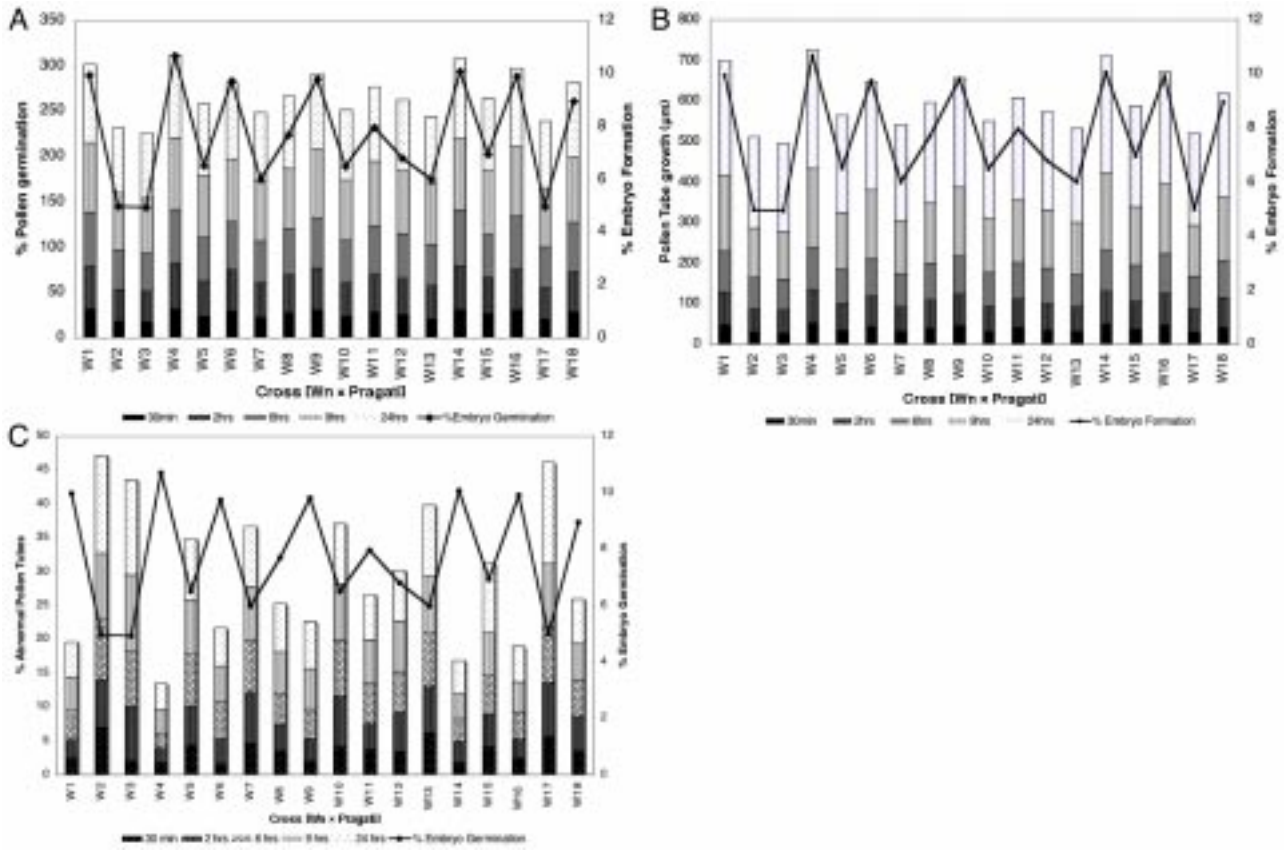

Fig. 1. A stacked plot of (A) per cent pollen germination, (B) pollen tube growth, (C) per cent abnormal pollen tubes at various time intervals after pollination in different wheat (Wn) x maize (Pragati) crosses. Overlaid as a line representation on the plot is embryo formation frequency (EFF) in percentage

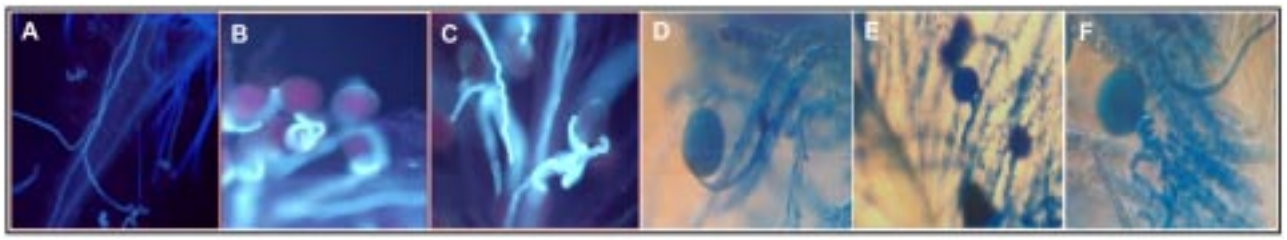

Fig. 2. Fluorescence and Normal Microscopy of pollen tube growth and abnormal development. (A) A normal pollen tube growing into stigmata (100X), (B) Coiling of pollen tube (200X), (C) Two pollen tubes growing from same pollen grain (200X), (D) Swelling of pollen tube tips (400X), (E) Pollen tube bifurcation (200X), (F) Twisting of pollen tubes (200X)

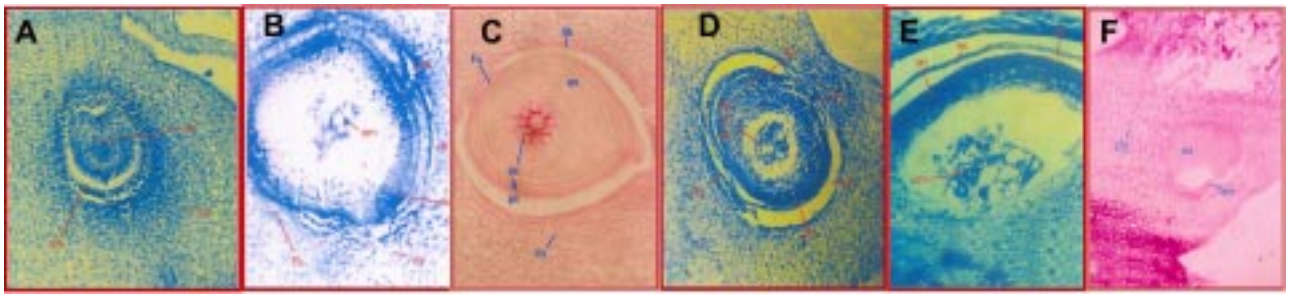

Fig. 3. Post-Fertilization studies on Wheat F_1W_3 (Dorade 5 × UP 2590), selfing and cross with Maize, using microtomy and light microscopy. Staining was performed with Coomassie brilliant blue (A, B, D & E) and Periodic Acid Schiff's reagent (C & F). (A) 8 h after selfing (100X), (B) 48 h after selfing(100X), (C) 8 h after selfing (200X), (D) 8 h after cross pollination (200X), (E) 48 h after cross pollination & (F) 48 h after cross pollination (200X) respectively. (ec – Egg cell, em – Embryo, en – Endosprem, es – Embryosac, oy – Ovary, ob – Obturator, ov – Ovule, Fu – Funiculus, pn – Polar nuclei)

genotype dependent, range of pollen tube growth in different genotypes seems to confirm the fact. Although this is matter of further study and to select efficient genotypes related to a particular trait as in our case it is cold tolerance. Gao *et al.* [15], O'Donoughue *et al.* [17], Jagadev *et al.* [8] reported incompatible interactions in various different wide cross hybridizations and analyzed different secretions in those crosses.

Thus, these parameters are important characters that directly or indirectly affect the frequency of embryo formation and seed formation frequency. Combinations of Druchamp with UP 2425 and UP 2590 showed best results in their wide cross with maize. Both crosses displayed high pollen germination, pollen tube growth and thus high rates of seed and embryo formation. Therefore, these parameters are helpful in screening best cross combinations of wheat and maize within two to three days, and saving time and resources. Finally, these crosses are best and could be picked up for further production of wheat haploid embryos and doubled haploid population for QTL mapping of cold tolerant traits in wheat.

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