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ORIGINAL PAPER

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CROSSING METHOD FOR THE FURTHER GENETIC IMPROVEMENT OF *Silybum marianum* (L.) Gaertn.

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ABSTRACT

Silybum marianum (milk thistle) is among the top selling herbal products in the U.S., in Italy and in other countries. Despite its economic importance as an officinal and medicinal species, only few improved genotypes exist and the species is still marked by traits that are typical of non-domesticated plants. Based on this evidence, further genetic improvement is required for the complete exploitation of milk thistle as a crop species. The first step in order to allow further breeding programmes of the species is the setup of a reliable crossing protocol. The present paper, after a brief review of *S. marianum* flower anatomy, describes a crossing method for this plant. The correct phenological stage for emasculation is BBCH growth stage 54. If manual pollination is repeated two days after emasculation, a significant increase of F1 fruits can be obtained. By utilizing the proposed protocol, an average 70–90 florets per flower head are emasculated and percentage of success (F1 fruits harvested) usually ranges between 27 and 70%. The proposed crossing protocol will constitute an important tool in order to allow the further genetic improvement of this species.

Key words: milk thistle breeding, crossing protocol, emasculation, controlled pollination, domestication

INTRODUCTION

Silybum marianum (L.) Gaertn. (common name milk thistle) is an annual or biannual species belonging to the *Asteraceae* family. *S. marianum* is a diploid species (2n = 34) [Tutin et al. 1976] and it was described to be autogamous with an average outcrossing rate of 2% under field conditions [Hetz et al. 1995]. The species is native to southern Europe, Asia Minor and northern Africa and it has been naturalized in North and South America, Australia and New Zealand [Groves and Kaye 1989, Morazzoni and Bombardelli 1995, Martin et al. 2000, Carrier et al. 2002].

S. marianum has been utilized as a medicinal plant for more than 2000 years [Morazzoni and Bombardelli 1995]. The medicinal properties of *S. marianum* are determined by its ability to accumulate the complex of bioactive flavonolignans, referred to as silymarin, in the fruit coat [Wagner et al. 1974, Cappelletti and Caniato 1984]. At present, the species is grown as a medicinal plant in Europe and in Asia [Andrzejewska et al. 2015] and it is among the top selling herbal products in the U.S., in Italy and in other countries [ISMEA report 2013, Smith et al. 2016].

Other than silymarin, the *S. marianum* fruit also contains valuable products such as oil (from 26.7 to 31.7% of fruit dry wt^{-1}; Martinelli et al. [2016]) and protein (16.5% of fruit dry wt^{-1}; Andrzejewska et al. [2015]) and it was tested as a potential commer-

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cial seed crop in Canada and New Zealand [Carrier et al. 2002, Martin et al. 2006]. Its possible non-medical uses were recently reviewed [Andrzejewska et al. 2015].

Despite the increasing interest in *S. marianum* as a multipurpose crop and its economic importance as an officinal species, only ten improved genotype/cultivars exist [Alemardan et al. 2013]. The species is still marked by traits that are typical of non-domesticated plants: fruit scattering at maturity, asynchronous flowering, spiny leaves, variable yield stability and quality [Martinelli et al. 2016]. As regard as both the plant morphology and the valuable fruit constituents, a wide phenotypic variability between accessions of different origins was observed but improved cultivars for these traits have not been released, yet [Ram et al. 2005, Martinelli et al. 2016]. Hence, further genetic improvement is mandatory for the complete exploitation of milk thistle as a crop species [Alemardan et al. 2013, Andrzejewska et al. 2015].

A precise and reliable crossing technique is fundamental in order to design breeding protocols for the development of improved cultivars taking advantage of the available genetic variability. Hetz et al. [1995], while assessing the degree of allogamy of the species, provided details about the utilized crossing technique, but a precise description of a complete crossing protocol is still lacking.

The aim of this study, along with a brief review of *S. marianum* flower anatomy, is to provide a specific crossing method supported by pictures and detailed description of the different operations. The crossing protocol will use the BBCH growth stages (Biologische Bundesantalt, Bundessortenamt and Chemische Industrie) [Martinelli et al. 2015] for the precise identification of the phenological stages at which each operation should be performed.

MATERIALS AND METHODS

During the years 2016 and 2017 a total of 34 controlled crossings (emasculated and pollinated flower heads) were performed between genotypes characterized by different silymarin chemotype and fruit fatty acid profile. In both years, the plants were sown in October at the experimental farm of Budrio (Bologna, Italy) and the crossings were performed during the first half of May.

In order to evaluate the importance of repeated pollinations, 17 flower heads were manually pollinated both immediately and two days after floret emasculation, the remaining 17 heads were pollinated only immediately after emasculation. After fruit ripening, the number of obtained fruits in each flower head was counted.

Nine flower heads were emasculated at different developmental stages according to the degree of pistil pigmentation. After emasculation these flower heads were isolated, with white nonwoven fabric, but not pollinated, this in order to better understand when, during flower head development, self-pollination takes place.

RESULTS AND DISCUSSION

Capitulum, flower and fruit: anatomy, phenology and natural pollination process

Like in other *Asteraceae* species, in *S. marianum* the flowers (called florets) are organized in inflorescences called flower head or *capitulum*. Flower heads are discoid, large with bracted peduncles, and involucre of ovoid to spheric shape. Phyllaries are graduated in 4–6 series. Tips of outer and middle spreading phyllaries are of lanceolate to ovate shape, spiny-fringed and tipped. The receptacle is flat, epaleate, bristly, and white coloured. In florets, the corolla is slender with long tube, throat that became abruptly wider and present linear lobes (fig. 1A and 2A). Anthers form a tube that surround the style during the first developing phase (fig. 1, 2B). Anthers' basis is sharply short-sagittate and tips are oblong. The style tip is long-cylindric distal to slightly swollen node. Style branches are very short (fig. 1B) [Keil 2017]. The colour of the different floret parts changes during floret development. The corolla is white at the end of the inflorescence emergence (BBCH 54; fig. 2C), it turns to light violet at the beginning of flowering

(BBCH 63), and to violet at the end of flowering (BBCH 69). On the contrary, anthers are deep violet at the end of inflorescence emergence (BBCH 54; fig. 2B) and the intensity of colour gradually decreases during flowering. Style is completely white at the end of inflorescence emergence (BBCH 54; fig. 2C) when it is completely enclosed in the anther tube, thereafter, the style gradually turns violet. Style pigmentation process starts just before the exit of the style from the anther tube and proceeds basipetally. At the end of flowering, the style appears completely violet.

As far as fruit anatomy is concerned, at maturity the fruit is ovoid, slightly compressed and glabrous. Pappus is composed of many flat, minutely barbed bristles that fall in a ring [Keil 2017].

As regard as the time span between the different phases of the flowering process, in each flower head an average of 5 days is needed between the beginning and the end of the flowering (BBCH 60-69) [Dodd 1989]; thereafter, usually 21 to 23 days are needed between the end of the flowering and the beginning of the seed dispersal process (BBCH 69-88) (author's personal observation).

As for the timing of floret development, in each flower head the first part to develop is the ring of florets localized on the outer part of the receptacle (fig. 3B), subsequently flowering proceeds in a centripetal manner.

The number of florets in each flower head changes according to capitulum size. A higher number of florets are present in the central capitulum, in the capitulum of branches of lower order, and generally in more vigorous plants. Capitula of second order branches often contain more than 300 florets.

As for pollination, despite flower heads are continuously visited by pollinators, *S. marianum* was described to be autogamous with an average outcrossing percentage of 2% [Hetz et al. 1995]. The observation was confirmed by three relevant facts. Firstly, isolated flower heads always develop a complete fruit set; secondly, that no inbred depression was observed after continuous selfing; thirdly, different lines, recurrently multiplied nearby each other, remain stable even if non insulation is provided [Hetz et al. 1995, author's personal observation]. As a rule, the selfing process takes place with high frequency probably because when the tip of the style exits from the anther tube it is always completely covered by pollen and for the fact that style branches are very short (fig. 4A). Therefore, despite previous studies had described *S. marianum* as an allogamous species [Heinz 1987, Rasic et al. 2009], we can conclude, in accordance with the measurements performed by Hetz et al. [1995], that the species is mainly autogamous.

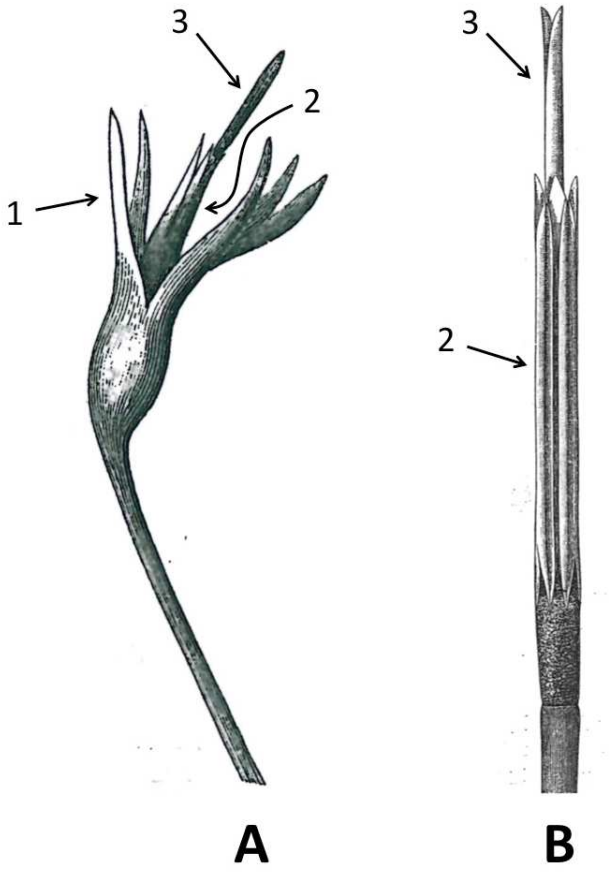

Fig. 1. Drawings representing a single flowering floret (A) and the detail of a floret without corolla (B). 1. corolla, 2. anthers, 3. style. Modified from Engler and Prantl [1897]

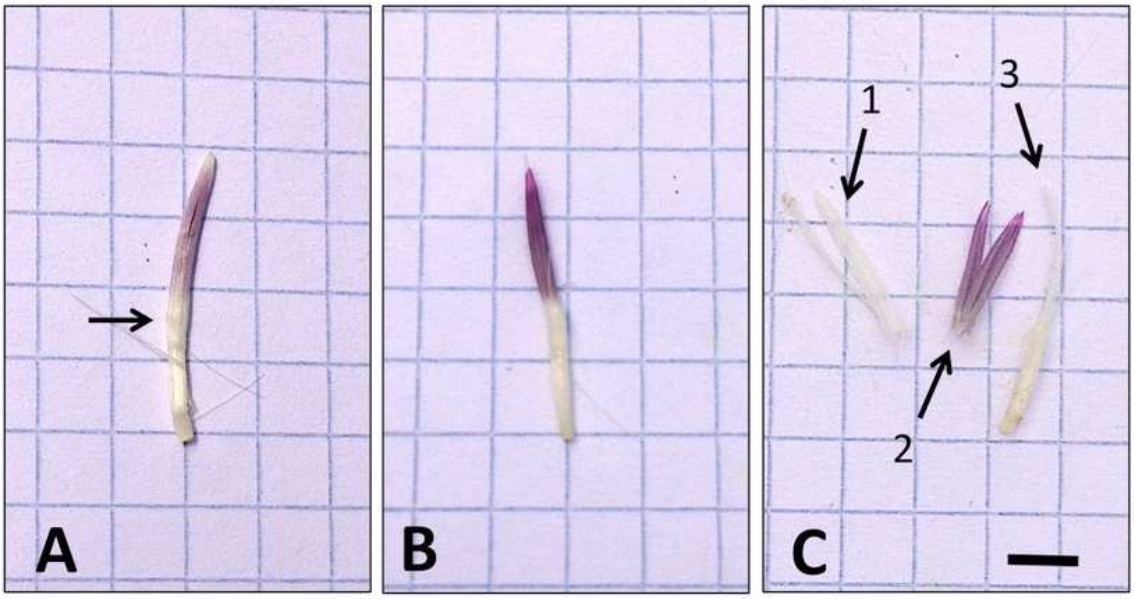

Fig. 2. Pictures showing a floret sampled from a flower head at BBCH stage 54 (A), the floret without corolla where the violet coloured anthers have been highlighted (B), and the dissected floret subdivided in: 1. corolla, 2. anthers, 3. style (C). The arrow in picture A indicates the level at which the corolla and the anthers should be detached with tweezers during the emasculation process. Bar = 4 mm

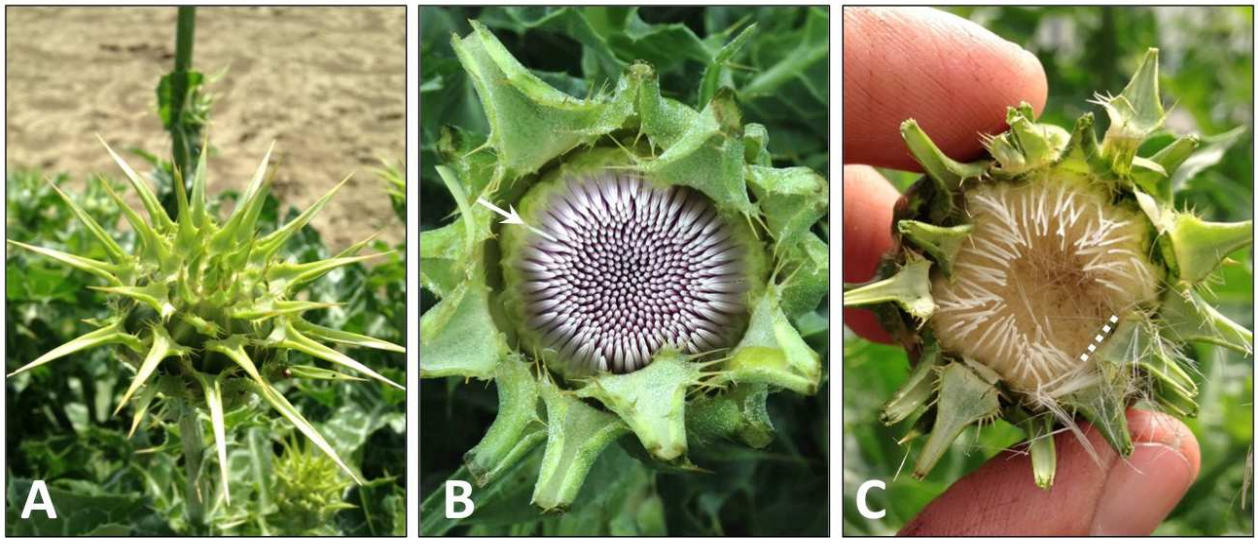

Fig. 3. Pictures of a *S. marianum* flower head during emasculation procedure. A. a flower head ready for emasculation (BBCH stage 54). B. flower head after the removal of phyllaries, the arrow indicates the first emasculated floret. C. flower head at the end of emasculation procedure where the ring of emasculated florets is visible; the dashed line indicates the side of the flower head from which it was possible to easily remove the innermost florets with the aid of tweezers

Inflorescence choice and emasculation procedure

The first step of the crossing procedure concerns the choice of the flower head to emasculate. When available, it is better to select big flower heads located on branches of lower order given that they are easier to be handled. The flower head should be at the end of inflorescence emergence stage (BBCH 54) with no florets visible at the centre of the inflorescence. Nevertheless the flower head should not be too immature because in this case the pistils will be fragile and they will be easily damaged during emasculation procedure. Usually the right moment for emasculation is when, at the centre of the flower head, the last layer of phyllaries still covers the florets but it is about to open itself (fig. 3A).

Once the inflorescence is chosen, it is important to cut the spiny bracts along the peduncle, the long spines on outer phyllaries, and to cut the distal part of inner phyllaries with small scissors in order to completely discover the florets (fig. 3B). As for the inner and tender phyllaries instead of cutting it is easier to detach them by pulling them out with the aid of a pair of tweezers.

The florets localized at the same distance from the centre of the receptacle are at the same developmental stage and the outer florets are always the most mature. Emasculation should be performed on 70–90 florets localized along a ring at the periphery of the receptacle (fig. 3C) of each flower head selected as described above. Each single floret should be emasculated by eliminating both the corolla and the anther tube (fig. 2). This operation is possible with the aid of fine tweezers by pinching and pulling where the immature floret stars to be wider (fig. 2A, arrow). During this operation it is fundamental to remove completely the pigmented portion of the anther tube that matches with the tissue that is responsible for the pollen production. After emasculation, the naked pistil should be completely white to slightly violet at the apex. Hetz [1995] reported that, in order to prevent accidental self-pollination, pistils should be white and not completely violet. We observed that if just the first mm (ca) of the pistil starts to turn violet and no pollen has been observed detaching from the anther tube during emasculation, self pollination has not taken place yet. On the contrary, at this stage the pistils are stronger and less easily damageable during emasculation procedure in comparison to completely white ones.

After emasculation the florets still attached to the centre of the receptacle have to be eliminated. This is to be performed by pulling the florets from the base, near the point of attachment to the receptacle, with tweezers. To make this operation easier and in order to avoid accidental damages to the emasculated pistils, we suggest to leave one small sector of the flower head with no emasculated pistils, this will make it easier to detach inner florets working with tweezers from that side of the inflorescence (fig. 3C).

Pollination procedure

Pollen should be collected from flowering flower heads (BBCH stage from 60 to 69) in the morning otherwise bees and other pollinators will take away most part of it during the day. Alternatively, some flower heads on the "father" plant can be protected from insects with the aid of a small net bag of suitable mesh. Anthers collected in the morning can be stored in glass vials at room temperature for further utilization during the day.

Mature pollen is light violet coloured and is clearly visible at the top of the anther tube of each flowering floret (fig. 4A). In order to collect pollen, we usually clamp few mature florets with tweezers and we cut them with scissors (fig. 4B). The obtained bunch of mature florets can be immediately used like a small brush in order to pollinate the emasculated flower head.

Pollination is usually performed immediately after emasculation of the "mother" flower head. Nethertheless, the number of produced fruits significantly increase if pollination is performed both immediately after emasculation and two days after, this results in almost double amount of harvested F1 fruits ($p < 0.05$; $n = 17$). During the pollination procedure it is fundamental to pollinate accurately the tip of the styles where the stigma, i.e. the pollen-receptive structure, is located.

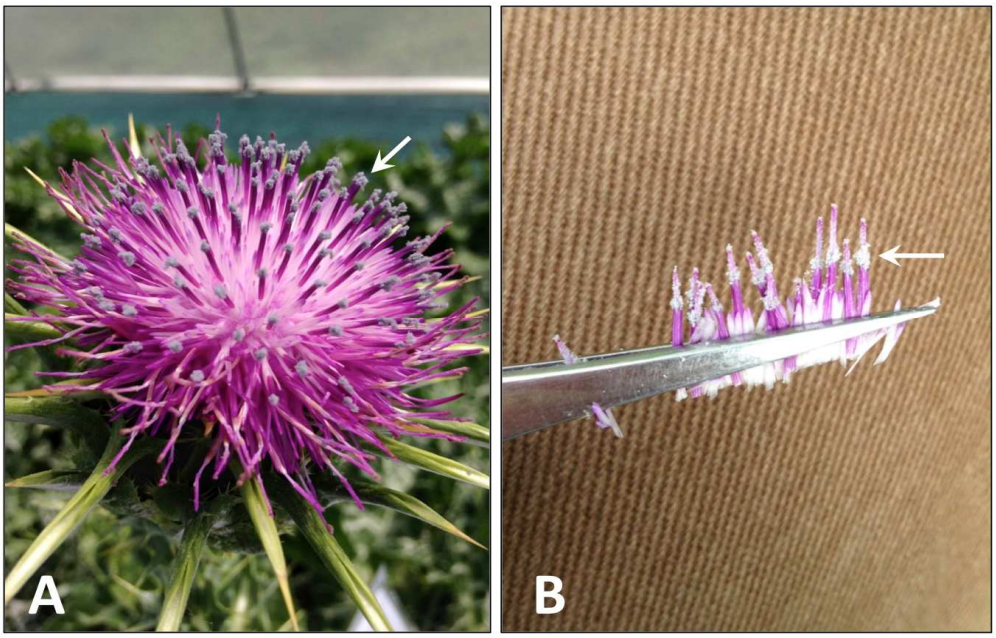

Fig. 4. Pictures showing the localization of pollen during flowering. A. flower head at flowering (BBCH 67), the arrow indicates a clump of mature pollen at the top of a floret. B. a group of mature florets ready to be used for the pollination procedure, the arrow indicates mature pollen on a style

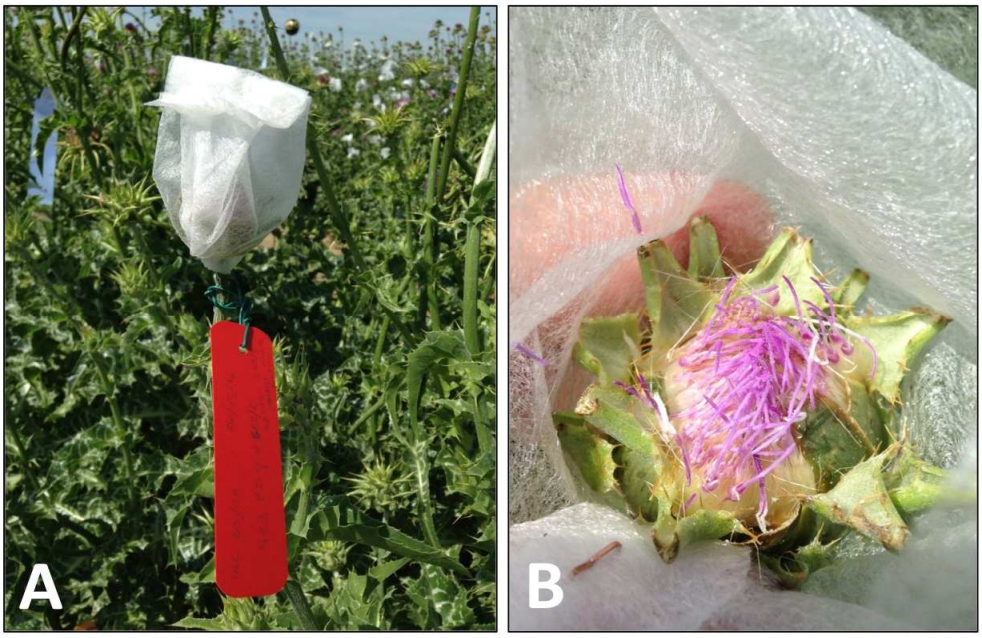

Fig. 5. Pictures showing a flower head tagged and covered with nonwoven fabric after the crossing procedure (A) and the styles two days after emasculation when the pollination procedure is usually repeated for the second time (B)

After manual pollination the flower head is immediately covered with a piece of white nonwoven fabric closed with the aid of a stapler (fig. 5A) in order to avoid further contamination from external pollen grains. Each cross is conveniently identified with a tag indicating the date of emasculation, of pollination and the parental lines utilized (fig. 5A). In figure 5B a flower head is showed two days after emasculation at the moment of the second pollination procedure.

Harvest of F1 fruits

Mature fruits can be conveniently harvested from crossed flower heads after one month from first pollination. In our experience, if the here described crossing method is applied, the percentage of harvested F1 fruits range between 26 and 70% (average 44.44% ±18.45 SD; n = 6) of emasculated florets.

CONCLUSIONS

The proposed crossing method allows the production of ca 35–40 F1 fruits per cross if the pollination procedure is repeated two times. As for the duration of the procedure, the entire crossing method, from the beginning of the flower head emasculation to pollination, usually lasts 40–45 minutes depending on the number of emasculated florets.

In order to fully exploit *S. marianum* as a crop species, further genetic improvement is mandatory. The first objective of this process should be the identification of lines with reduced/absent fruit dispersal at maturity in order to significantly reduce yield losses and to minimize the presence of *S. marianum* as a weed in crop rotation. At this moment both a mutagenized *S. marianum* population and *S. marianum* wild accessions have been screened in at CREA-CI (Bologna, Italy) order to identify key lines that will permit both the complete domestication of the species and the identification of other traits of interest (e.g. modified silymarin or fruit fatty acid profile).

The here proposed crossing method will be an important tool for the further breeding of the selected lines in order to allow the genetic improvement of this promising species.

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