UV induced visual cues in grasses

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Grasses are traditionally considered as wind pollinated, however, field observations confirmed frequent insect visits to grass flowers, suggesting insect pollination. Fruit and seed predators inflict heavy losses to cereals and millets during their growth, maturation and storage. The actual factors guiding insects and predators to grass flowers, fruits and seeds are not clear. Here, we report attractive blue fluorescence emissions on grass floral parts such as glumes, lemma, palea, lodicules, staminal filaments, pollens and fruits in ultraviolet (UV) 366 nm, whereas the stigmatic portions were not blue, but red fluorescent. We characterized the blue fluorescent constituent in grass reproductive structures as ferulic acid (FA). Fluorescence spectra of blue-emitting grass floral, seed extracts and isolated FA on excitation at 366 nm showed their emissions at 420–460 nm. We propose these FA-based blue fluorescence emissions in grass reproductive structures as visual cues that attract pollinators, predators and even pests towards them.

Results

Grasses are monocot family Poaceae include cereals (rice, wheat, maize), millets (bajra, ragi), bamboos and fodders. They are vital food sources for humans and animals. The lignocellulosic biomass of grasses is considered as a renewable feedstock for biofuels. Though grasses are known to be wind pollinated, recent studies reported bee and insect visits to bamboo flowers in south America, south India and central China. Moreover, insect visits to grass flowers (florets) were observed in habitats where wind is negligible. Our own field observations on rice, maize and bamboos and other reports provide sufficient documentation of bee, insect and pest visitations to grasses at flowering and fruiting stages. These reports led to strong suspicion that floral visitors might play a role in pollination. Grass flowers are small but numerous in clusters, and are not showy compared to orchids or roses. With no attractive colour, nectar or scent, and pollen as the only reward, it is not clear how bees and insects are enticed to grass flowers. Similarly, rodents and other animals inflict heavy losses to grass fruits and seeds (food grains), and the factors guiding these predators to them are unclear. Here, we show that FA-based attractive blue fluorescence emissions from grass flowers, fruits and seeds play a potential role in insect/animal attraction.

Like in other flowering plants, the floral parts in grasses such as glumes, lemma and lodicules of grasses Oryza sativa (rice), O. rufipogon (Griff. (red rice), Triticum aestivum (wheat), Zea mays (maize), Sorghum bicolor (L.) Moench (sorghum, 'cholam'), Sorghum bicolor (L.) Moench (sorghum, 'kattucholam'), Eleusine coracana (L.) Gaertn. (ragi, finger millet), Pennisetum glaucum (L.) R. Br. (bajra), Ochlandra travancorica (Bedd.) Gamble, Bambusa pallida Munro and Melocanna baccifera (Roxb.) Kurz, both in flowering and fruiting stages at UV 366 nm (Fig. 1b–j, l, n, p). Lodices, with only known role in opening of florets, were strongly blue fluorescent (Fig. 11). Staminal filaments were intensely blue fluorescent in O. travancorica, B. pallida and M. baccifera (Fig. 11), but relatively less fluorescent in O. sativa, O. rufipogon and T. aestivum. Pollen grains of O. travancorica, B. pallida and M. baccifera were strongly blue fluorescent (Fig. 1j). When fluorescent pollens were dusted off, their anthers appeared mild red with chlorophyll emissions (Fig. 1j, l, p). Anthers in O. sativa, O. rufipogon, T. aestivum, Z. mays, E. coracana and P. glaucum appeared faintly blue fluorescent. Pollens in these tiny grass florets were relatively less fluorescent. Ovaries and styles in all grass species observed were blue fluorescent (Fig. 11). Stigmas were not blue fluorescent but mildly red (Fig. 11).

Unlike in other flowering plants, the floral parts in grasses such as glumes, lemma and palea do not fall after pollination, but remain as persistent protective structures in mature fruits (Fig. 1n). Glumes in O. sativa and O. rufipogon grains emitted intense blue fluorescence at UV 366 nm (Fig. 1f, g). Glumes of aborted spikelets were relatively less blue fluorescent compared to fruiting ones. In O. travancorica fruits, persistent lemma and palea were blue fluorescent at the outer surface and their inner unexposed surfaces were less fluorescent (Fig. 1n). Both
fruit outer cover and endosperms of these grasses were fluorescent, with inner (cut or crushed) portions showing significantly higher intensities of blue emissions (Fig. 1d, k). In *Z. mays*, the sheaths covering its cobs were strongly blue fluorescent with highest emissions from innermost sheaths (Fig. 1c). Common fodder grasses *Pennisetum polystachion* (L.) Schult. and *Axonopus compressus* (Sw.) P. Beauv. also showed similar blue fluorescence patterns on their relatively tiny floral and fruiting structures.
We isolated the blue fluorescent fractions from fresh grass specimens viz. *Z. mays* (leaves, male flowers, external bracts, internal bracts, lemma/palea/lodicule, immature seeds), *O. sativa* (leaves, spikelets, immature seeds), *E. coracana* (mature seeds), *O. travancorica* (leaves, spikelets, lemma/palea/staminal filaments/pollen) and *B. pallida* (leaves, spikelets, lemma/palea/lodicules, immature seeds) by alkaline hydrolysis and extraction (see Methods). Blue fluorescent fractions were also isolated from mature seeds (dry, stored) of *Z. mays*, *O. sativa*, *T. aestivum* and *B. pallida*. On HPTLC profiling at 366 nm, *Z. mays*, *T. aestivum*, *O. sativa*, *E. coracana*, *O. travancorica* and *B. pallida* extracts showed blue spots at retention factor (Rf) 0.54, exactly matching with FA (Fig. 2a, b). FA (blue) was the major spot in extracts of floral parts and seeds of these grasses. Leaf extracts of grasses showed multiple chlorophyll (red) signals in addition to FA (Fig. 2a). FA contents in leaves, floral parts and seeds of *Z. mays*, *O. sativa*, *T. aestivum*, *O. travancorica* and *B. pallida* extracts showed strong red chlorophyll signals at 366 nm, but the blue-emitting FA signals detected were very feeble (Fig. 1q, S1).

Blue FA spot was isolated in pure form from *T. aestivum* seed extract (see Methods) (Fig. 2b). On excitation at λ 366 nm, *T. aestivum* seed extract, isolated FA and standard FA showed fluorescence emission maxima in the blue (420–460 nm) region (Fig. 2c). But, *C. nutans* leaf extract showed strong red chlorophyll emissions at 655–695 nm. Both isolated and standard FAs showed overlapping UV absorption spectra with their maxima at 322 nm.

**Discussion**

We found grass inflorescences very attractive with UV-induced blue emission patterns. These blue emissions from critical floral parts of grasses are not known so far. UV induced floral emission patterns were reported as strong visual signals to pollinators in colourful dicot flowers of *Hypericum calycinum* (flavonoids) and *Mirabilis jalapa* (betaxanthin, betanin). Recently, we reported strong blue fluorescence emissions from prey traps of carnivorous plants *Nepenthes*, *Sarracenia* and *Dionaea* in UV14. Over a decade ago, we recorded bee (Apis, Halictus, Trigona, Braunsaip, Ceratina) visits to six woody bamboos (Ochlandra travancorica, *O. ebracteata*, *O. scriptoria*, *Bambusa bambos*, *B. vulgaris*, *Bambusa sp.*) in the same geographical fields in south India as described in this study15. Since then we observed similar bee visit patterns to the bamboos in our Institute Bambusetum (N 08° 45.268′–43.4′ E 077° 01.429′–59.4′). Our recent observations clearly demonstrate bee visits (and foraging) on bamboos (Fig. 1m, o). These bees were netted and identified as *Apis dorsata* Fabricius (largest), *A. cerana indica* Fabricius (medium) and *Trigona iridipennis* Smith (small, in flight) on *O. travancorica* (Fig. 1m) and *Halictus taprobane* Cameron on *B. pallida* (Fig. 1o). Our data (Fig. 1m, o)16 and other previous studies6–8 found bees and other visitors only on the main stages of bamboo flowers, and reported pollen collections by them. These visitor activities were not observed in the female stages of their flowers17. Blue signaling from the male stages of flowers and pollens observed in this study (Fig. 1j, l, p) is coinciding with the visitor hits and pollen collection patterns (Fig. 1m, o) reported earlier18.

We also observed distinct blue emissions at UV 366 nm from the endosperms of the cereals, millets and bamboos (Fig. 1d, k). Seed predators or dispensers like birds, rats and other small mammals could see these UV induced blue fluorescence emissions18–24. Rats are known fruit predators of bamboos25,26. Rodent and other predation losses of cereals and millets in seedling, grain and warehouse stages are hugely significant27. Most nocturnal species, active in late evenings and in the darkness of the night, have adapted sensitive vision aiding them to find their food and mates22,25,26. Even low levels of UV in nocturnal conditions could lead to blue emissions from grass flowers, fruits and seeds. These fluorescence emissions from grass reproductive structures are in the blue region of the visible spectrum, which is the best detectable range for arthropods, birds, rats and other small mammals16–22. These blue emissions from grass flowers and seeds could attract seed dispensers, predators and even insect pests towards them16–20. Under UV, the reproductive structures of grasses are truly ‘showy’.

Our UV induced fluorescence emission (Fig. 1), HPTLC (Fig. 2a, b) and fluorescence spectral (Fig. 2c) data clearly showed that FA is causing the blue emissions from the floral parts, fruits and seeds of grasses. The role of cell wall bound FA as distinct visual cues (blue emissions) in vital reproductive structures of grasses is not known so far. On estimation, in maize and rice, FA contents in floral portions were higher compared to their leaves, up to 3.23 times in internal bracts of maize cob compared to its leaves (maize, male flowers: leaves 2.34, cob external bracts: leaves 1.77; rice, spikelets: leaves 1.42, immature seeds: leaves 1.16) (Table 1). Dry seeds of both maize (seeds: leaves 0.80) and rice (seeds: leaves 0.06) showed relatively lower FA contents compared to their fresh leaves (Table 1).

Previous studies found graminaceous cell walls as rich natural sources of FA (up to 3.0%, dr. wt. in maize bran), and it is mostly bound in the form of ferulate-polsaccharide-lignin complexes22–25. FA contents in rice grains29 and rice endosperm cell walls28,30,31 were estimated as 0.0061–0.0362 (%, dr. wt.) and 0.91 (%, dr. wt.), respectively. In bamboos, floral portions of *O. travancorica* (spikelets: leaves 0.44, flowers: leaves 0.28) and *B. pallida* (spikelets: leaves 0.68) showed a lower content of FA compared to their leaves (Table 1). Bamboo flowers are relatively big, and FA-based emissions are highest in their staminal filaments and tiny lodicules (Fig. 11). Being taller plants (3 to 25 m), bamboo floral emissions in fields are at elevated levels, and intense enough to attract insects and bees onto them.

Visibly, floral portions, fruits and seeds are emitting strong blue at UV 366 nm in all grasses, including bamboos (Fig. 1b–l, n). Pollen grains in bamboos (Fig. 1j), the main reward for insects and bees, were strongly blue-emitting in UV. Grass leaves showed significant red emissions (multiple bands) of chlorophyll (Fig. 2a, S1), which absorbs sunlight leading to photosynthesis. Fluorescence from grass leaf matrices at UV 366 nm are mixed visual hues of chlorophyll red and FA-based blue emissions. Though grass leaves showed comparable contents of FA, mixing of dominant red and blue emissions results in less intense blue hues on leaf matrices in UV (Fig. 1e, f, n). Chlorophyll contents are very low in grass floral portions, fruits and seeds (Fig. 2a). *O. sativa* (Fig. 2a, t6), *O. travancorica* (Fig. 2a, t8) and *B. pallida* spikelets (Fig. 2a, t10) showed faint red chlorophyll emissions. While most floral portions were blue-emitting, stigmatic portions and anthers of *O. travancorica* (Fig. 1j, l) and *B. pallida* (Fig. 1p) were found emitting mild red fluorescence at UV 366 nm. Blue emissions from grass reproductive structures for the most part are not mixed with chlorophyll or other emissions. Red chlorophyll bands were clearly visible in extracts of dicot leaves of *C. nutans* and *S. nodiflora* (Fig. 1q, S1). These dicot leaf extracts showed only very weak FA bands (Fig. S1). At UV 366 nm, relative FA/chlorophyll contents in dicot leaves, grass leaves and grass reproductive structures are reflected in their fluorescence emissions as red (Fig. 1q, S1), red-blue mix (Fig. 1e, f, n) and blue (Fig. 1b–l, n, p), respectively (Fig. 2a).

Visual cues from grass reproductive structures (blue) and leaves (red-blue) in UV effectively reflect the dominance of FA versus chlorophyll based emissions (Fig. 1a, 2a). Most insects, bees (*Apis, Halictus, Trigona, Braunsaip, Ceratina*) and seed predators have their three vision maxima around 340 nm (UV), 430 nm (blue) and 535 nm (green)26. But, UV-induced red chlorophyll emissions are at λ 655–695 nm26,32. Flower and fruit visitors, therefore, cannot see the
red emissions (as red) from grass leaves, unless they possess extra red receptors. Similarly, stigmatic portions in grasses, particularly in bamboos, have mild red emissions, and insect visitations were not observed on them. But, blue emissions (420–460 nm) from grass reproductive structures (Fig. 1, 2a, b) are within the vision maxima (430 nm, blue) of insects, bees and seed predators. Faint UV induced red-blue mix emissions from grass leaves (Fig. 1e, f, n) and absence of insect visitations on mild red emitting stigma of bamboo florets are indications that insects and predators are directed towards FA-based blue-emitting vital reproductive structures.
Alkaline hydrolysis and extraction of blue fluorescent fractions from grasses, estimation of ferulic acid

Table 1 | Ferulic acid (FA) contents estimated in grass reproductive structures

| Grass specimen             | FA content (% ± SD, n = 4, fresh wt.) |
|----------------------------|---------------------------------------|
| Zea mays                   |                                       |
| Leaves                     | 0.1162 ± 0.0043                       |
| Male flowers               | 0.2721 ± 0.00                        |
| External bracts            | 0.2057 ± 0.0037                      |
| Internal bracts            | 0.3577 ± 0.0025                      |
| Lemma/palea/lodicule      | 0.1129 ± 0.0055                      |
| Immature seeds            | 0.0832 ± 0.0003                      |
| Mature seeds (stored)      | 0.0932 ± 0.0015*                     |
| Oryza sativa               |                                       |
| Leaves                     | 0.2574 ± 0.00                        |
| Spikelets                  | 0.3654 ± 0.0069                      |
| Immature seeds            | 0.2998 ± 0.00                        |
| Mature seeds (stored)      | 0.0155 ± 0.00                        |
| Triticum aestivum          |                                       |
| Mature seeds (stored)      | 0.0304 ± 0.00                        |
| Ochlandra travancorica     |                                       |
| Leaves                     | 0.2883 ± 0.0052                      |
| Spikelets                  | 0.1278 ± 0.00                        |
| Lemma/palea/staminal pollen | 0.0796 ± 0.0032                 |
| Bambusa pallida            |                                       |
| Leaves                     | 0.2270 ± 0.0004                      |
| Spikelets                  | 0.1543 ± 0.0007                      |
| Mature seeds (stored)      | 0.0984 ± 0.0010*                     |

* % ± SD, n = 4, dry wt.

(Fig. 1j, l, m, o, p). Grass floral parts that appear thin and hyaline in daylight, especially edges of glumes, lemma, palea, showed strong blue emissions at UV 366 nm, proportional to their FA contents (Fig. 1o, p). Significantly, FA provides photoprotection to vital cellular components in grass reproductive structures by absorption of damaging UV radiation and emitting as harmless blue patterns. In conclusion, our study found floral parts, fruits and seeds of grasses very 'attractive' in UV induced fluorescence emissions. We propose these FA-based blue emissions from grass reproductive structures as enticing visual cues to pollinators (bees, other insects), seed dispersers (birds) and predators (birds, rats). This study thus provides more evidence that insect pollination is possible in grasses. Pollen transfer studies could further confirm entomophily in grasses. These blue emissions could also act as signals attracting insect pests to grains of cereals and millets. Also, we showed that, FA-based emissions are playing a crucial role in plant-animal interactions. Our findings could also help redefining the functions of grass floral parts and better understanding their morphology. Future studies on signaling molecules and defense mechanisms in grasses could lead to the discovery of novel molecular or fluorescence-based pest, weed control methods.

Methods

Grasses, UV emissions. Floral parts, fruits, seeds and leaves of six cereals, Oryza sativa L. (rice), O. rufipogon Griff. (red rice), Triticum aestivum L. (wheat), Zea mays L. (maize), Sorghum bicolor (L.) Moench (sorghum, ‘cholam’), Sorghum bicolor (L.) Moench (sorghum, ‘kattuchelam’), two millets, Eleusine coracana (L.) Gaertn. (ragi, finger millet), Pennisetum glaucum (L.) R. Br. (bajra), three bamboos, Ochlandra travancorica (Bedd.) Gamble, Bambusa pallida Munro, Melocanna baccifera (Roxb.) Kurz and two common fodder grasses, Pennisetum polystachum (L.) Schult., Asnodon compressus (Sw.) P. Beauv. were collected from six locations in south India, (i) Jawaharlal Nehru Tropical Botanic Garden and Research Institute Bambusetum, Palode, (ii) College of Agriculture, Vellayani, (iii) Cropping System Research Centre, Thiruvananthapuram, (iv) Peringamala, Thiruvananthapuram, (v) Meenakshipuram, Travancore (vi) Karamana, (vii) Peringamala, Thiruvananthapuram, (viii) Meenakshipuram, Travancore (ix) Jawaharlal Nehru Tropical Botanic Garden and Research Institute Bambusetum, Palode, (ii) College of Agriculture, Vellayani, (iii) Cropping System Research Centre, Thiruvananthapuram, (iv) Peringamala, Thiruvananthapuram, (v) Meenakshipuram, Travancore (vi) Karamana, (vii) Peringamala, Thiruvananthapuram, (viii) Meenakshipuram, Travancore

Isolation of ferulic acid from Triticum aestivum seed extract (15 mg) in 1:1 water: methanol (400 µl) was applied on a silica gel plate 60F-254 (20 x 10 cm, 0.2 mm thickness, E. Merck, Darmstadt, Germany) as a band in preparative mode using HPTLC (CAMAG, Muttenz, Switzerland) and the plate was developed in 9:1 acetone-water and viewed at UV 366 nm. FA contents were calculated in extracts of leaves and reproductive structures of Z. mays, O. sativa, T. aestivum, O. travancorica and B. pallida were estimated by HPTLC-densitometry under identical conditions and developed spots were scanned at λ = 322 nm. Mobile phase for estimation was optimized to 9.5:0.5 acetone-water. Percentage FA contents (% ± SD, n = 1, based on fresh or dry weights) were calculated from peak areas, using the standard curve (y = 25111x + 107.8, R² = 0.996) with a linear relationship in the range 0.05 to 0.7 µg.

Ultraviolet absorption, fluorescence emission spectra. UV absorption spectra of FA isolated from T. aestivum seed extract and standard FA (Sigma-Aldrich, Bengaluru, India) were recorded on TLC Scanner 3 (CAMAG, Muttenz, Switzerland) and UV-1650PC UV-Visible spectrophotometer (Shimadzu, Kyoto, Japan). Fluorescence emission spectra of T. aestivum seed extract and FA (isolated and standard) dissolved in methanol were measured on a SPEX Fluorolog F112X spectrophotometer (Horiba Jobin, Edison, USA) at an excitation λ = 366 nm (Fig. 2c).

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