An experimental design to test the effect of wheat variety mixtures on biodiversity and ecosystem services

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The present document details how the Wheatamix consortium, inspired by ecological experiments exploring relationships between plant biodiversity and ecosystem functioning (e.g. the Jena experiment Weisser *et al*. 2017), selected bread wheat (*Triticum aestivum* L.) lines, phenotyped them across a range of functional traits and used this information to set up an experimental design able to unravel the effects of variety number and of the functional diversity and identity within variety mixtures for evaluating the impact of intraspecific crop diversity on a range of ecosystem services.

Wheat line selection

The Wheatamix project investigates the potential benefits of variety mixtures in the Paris basin wheat supply chain, and therefore focuses on varieties and lines adapted to the local climate. A consensus list of 57 wheat lines (Table 1) was thus settled on these grounds and to meet the expectations of agronomists, geneticists, phytopathologists and ecophysiologists of the group. This list is composed of i) 32 elite bread wheat varieties registered in the French catalogue, selected for their high yields under conventional farming, ii) 5 modern varieties bred for organic farming (OF), iii) 10 landraces resulting from farmers' mass-selection, cultivated in France in the early 1900es, and iv) 11 lines from an INRA-MAGIC multiparental and highly recombinant population (Thepot *et al*., 2015), adapted to Northern France. Due to the heterogeneity of information available for each variety and line, various criteria were used for this selection. The 32 elite bread wheat varieties were chosen on the basis of their wide use in the Paris Basin, and to ensure representativeness of the diversity for earliness, disease resistance or bread-making quality, using the available information in the variety catalogue (https://www.geves.fr/catalogue/). These varieties originated from the principal breeding companies. Some elite varieties were also included because they are often used in experimental research (Soisson, Apache, Caphorn for example). Landraces and organic varieties were selected on the basis of their wide use in low input or organic farming systems, and for their diversity of traits (plant height, earliness...). Finally, the 11 Magic lines were selected among 1000 available lines, based on their yield and to offer a broad diversity for earliness, plant height and genetic diversity (on the basis of SNP genotyping data, see Thepot *et al*., 2015).

Seeds from all varieties and lines were multiplied in 2013 and 2014 in INRA-GQE, Le Moulon, Gif-sur-Yvette, France, to provide sufficient seeds for the various experiments performed during the Wheatamix project.

Multi-trait phenotyping of the variety panel

The Wheatamix panel was phenotyped by different teams of the Wheatamix consortium to characterize both agronomic (*e.g.* yield, earliness and disease resistance) and ecological (*e.g.* specific leaf area and root absorption capacity of mineral N forms) traits. A matrix of 27 traits was used to summarize the functional diversity of the variety panel (Table 2).

Multi-trait classification of the variety panel and selection of a sub-panel

To select a subset of varieties representative of the diversity of traits in the panel of 57 varieties, different multivariate clustering analyses were performed on the 27 trait matrix. Missing value imputation, for a total of 19 missing values across all traits and varieties, was done assuming that there was no cluster, that the data came from a single multivariate normal distribution, and that missing values were distributed at random. Using the Pairwise method, a single covariance matrix was formed for all the data. Then each missing value was imputed by a method that is equivalent to regression prediction using all the non-missing values as predictors. The lines were classified using either hierarchical clustering (Ward method, using JMP Pro v.13 SAS software) and a cutoff was set to separate six functional groups of varieties. Two of them were excluded because they were not stable across statistical analyses.

The first branch in the tree roughly discriminates Landraces from modern varieties, and the landrace branch is itself strongly diverse, with 3 subtrees, as illustrated on the Fig. 1 with k=6. For practical reasons, we had decided to base our experimental design on only 4 groups and to obtain a comparable number of varieties /lines within the 4 selected subgroups, only one Landrace subtree was selected (the largest). We finally checked that the 4 remaining groups are stable, *i.e.* keep a similar composition when one of the 27 traits is removed (Jacknife) from the clustering analysis.

The four functional groups retained, hereafter c1-c4, are presented in Fig. 1. Functional group c1 includes 14 varieties (6 MAGIC lines and 8 elite varieties) that are generally sensitive to fungal diseases and have a low potential for soil exploration/exploitation, as characterized by root traits and capacity for absorption of nitrate and ammonium. This group contrasts with functional group c4, containing 17 elite varieties resistant to fungal diseases and with a higher potential for soil exploration/exploitation. Functional group c3 includes 9 varieties (5 landraces and 4 organic varieties) characterized by their slow growth but elevated aggressiveness regarding plant-plant competition. Finally, functional group c2 is composed of 8 varieties (7 elite and 1 organic varieties), without obvious pattern in terms of functional traits.

A sub-panel of four varieties within each functional group (Table 3), *i.e*. a total of 16 varieties, was selected, allowing manipulation of a reasonable number of varieties when choosing mixtures to set up the experimental design (next step). The choice of varieties within a functional group was constrained by seed availability. We also made sure that the 4-groups clustering remained robust after sub-sampling. This balanced contribution of each functional group maximized the overall functional diversity within the pool of 16 varieties. Among these, 7 were modern winter varieties, 5 were landraces or modern organic varieties, and 2 were INRA MAGIC lines.

The first three axes of a principal component analysis implemented on wheat traits of the 16 varieties selected (Fig. 2) extracted 59.2% of the total variance (26.2%, 18.2% and 14.8%, respectively). As expected, the 16 varieties were clustered consistently with their functional groups: c1, c2 and c4 appeared on the positive side and c3 on the negative side of axis 1. Besides, c1 and c2-c4 were distinguished on the second axis with c2 standing on the negative side and c4 on the positive side of the third axis. The wheat varieties (Altigo, Trémie, F426 and A22) of the c1 functional group were characterised by sensitivity to fungal disease and low flag leaf nitrogen content. Functional group c2 was composed of wheat varieties (Renan, Skerzzo, Midas, Alauda) with short root length, high level of NO_3^- absorption and high relative growth rate. The c3 functional group was composed of tall wheat varieties (Blé Autrichien, Hermès, Maxi, Ritter) that are not very aggressive and have high NH_4^+ absorption capacity. Finally, the c4 functional group contained varieties (Grapeli, Soissons, Arezzo, Boregar) with high specific root length, low relative growth rate and low NO_3^- absorption capacity (Table 3).

Variety mixtures used for the Wheatamix experimental plan

Using the 16 wheat varieties selected as detailed above, 72 different mixtures of varieties were created to explore a wide range of variety number and intraspecific functional diversity (Table 4). These include 24 different combinations of two varieties, 28 combinations of 4 varieties and 20 combinations of 8 varieties (Table 5). The 72 mixtures allowed us to explore extensively the gradient of functional diversity while using each of the 16 varieties in a perfectly balanced way at each richness level. For a given richness level (i.e. number of varieties), we varied the number of functional groups whenever possible (*e.g.* one or two functional groups in binary mixtures ; two to four functional groups in octonary mixtures ; see Table 5). This means that for each number of varieties in a mixture, the whole gradient of functional diversity was explored from mixtures with low functional diversity (only one functional group) to mixtures with low functional diversity (as many functional groups as possible given the number of varieties in the mixture). Three replicates of monocultures (here plots with a single variety) were used. However, as our objective was not to assess significant differences between pairs of mixtures but rather to quantify the effects of variety richness and functional diversity levels or functional group number, one replicate of each variety mixture was used.

Monocultures and mixtures were sown in Versailles (48°48'26"N 2°05'13"E) on November 2014. All plots were of identical size (10.5 m x 8.0 m) and divided into 6 sowing units of 1.75 m x 8 m. Each sowing unit consisted of 8 sowing lines spaced from each other leaving a 17.5 cm gap (Fig. 3). In autumn 2014, a seedbed was prepared by ploughing (20-30 cm deep) and the plots were sown in early November with monocultures or variety mixtures (see Table 4) at a density of 180 seeds m^{-2}. Each plot was buffered from adjacent plots or field edge by a 1.75 m-wide row of triticale (x Triticosecale) to standardize plot edge and possibly limit the dispersal of pathogen spores between neighbouring plots (Fig. 3). The crop was grown with a target yield of 60 q/ha (75 = French average national wheat yield in 2015). No insecticide and fungicide was used except for seed coating for which CELEST (0.2 $l.quintal^{-1}$ – Fludioxonil $25g.l^{-1}$) and SIGNAM (60 $g.quintal^{-1}$ – Cypermethryne 300 g.l^{-1}) were used. One spraying of an herbicide (Archipel® and Harmony Extra®) was performed on March 14, 2015. 160 kgN.ha^{-1} was used as compared to the estimated optimal amount of 180 kgN.ha^{-1} (Carlotti 1992). The fertilizer (ammonium-nitrate) was spread as follows: 40 kgN.ha^{-1} on March 5, 2015, 80 kgN.ha^{-1} on April 16, 2015, 40 kgN.ha^{-1} on May 11, 2015. All plots were harvested between the last week of July and the first week of August 2015.

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Table 1: List of the 57 varieties phenotyped in the Wheatamix project

Variety name	Functional group	Variety type	Variety name	Functional group	Variety type
Premio	c1	Elite	Sy Moisson	c1	Elite
Altigo	c1	Elite	Trémie	c1	Elite
Apache	c4	Elite	Tulip	c1	Elite
Arezzo	c4	Elite	A22	c1	MAGIC
Arlequin	c4	Elite	A160	c1	MAGIC
Attlass	c4	Elite	A208		MAGIC
Barok		Elite	A210		MAGIC
Boregar	c4	Elite	F236		MAGIC
Caphorn	c4	Elite	A243		MAGIC
Fanion	c4	Elite	A248		MAGIC
Farmeur	c1	Elite	A398	c1	MAGIC
Flamenko	c4	Elite	F426	c1	MAGIC
Folklor	c4	Elite	A446	c1	MAGIC
Goncourt	c1	Elite	A490	c1	MAGIC
Grapeli	c4	Elite	Blé Autrichien	c3	Landrace
Isengrain	c4	Elite	Rouge de Bordeaux		Landrace
Koreli	c2	Elite	Noé		Landrace
Lyrik	c4	Elite	Barbu de Champagne	c3	Landrace
Midas	c2	Elite	Alauda	c2	Organic
Odyssee	c4	Elite	Hermès	c3	Organic
Pakito	c1	Elite	Karneol	c3	Organic
Quebon	c4	Elite	Ritter	c3	Organic
Bermude	c4	Elite	Maxi	c3	Organic
Renan	c2	Elite	Prince Albert	c3	Landrace
Rubisko	c4	Elite	Rouge du Roc		Landrace
Skerzzo	c2	Elite	Saint Priest		Landrace
Sogood	c2	Elite	Sixt sur Aff	c3	Landrace
Soissons	c4	Elite	Royo de Pamplona	c3	Landrace
Solehio	c2	Elite			

Elite: modern commercial variety, registered on the seed market for conventional agriculture. **Organic**: modern commercial variety registered for Organic Farming. **Landrace**: old traditional variety that evolved over decades and adapted locally under the unconscious selection of farmers, and was commonly cultivated till 1930th. **MAGIC**: inbred lines developed from a multiparental (60 parents) and highly recombinant INRA population.

Table 2: Traits measured on the 57 varieties of bread wheat

	Traits	Unit	Meaning	Stage/age of the plant	Growth conditions	Location and year of measurements
Growth and allocation	SRR	No dimension	Shoot root ratio	8 weeks	Greenhouse conditions in 2L pots with sand and hydroponic solution, 3 plants per pot	Lyon, 2014
	RDMC	$mg.g^{-1}$	Root Dry Matter content			
	RGR	$mg.day^{-1}$	Relative Growth rate			
Nutrients contents and nitrogen cycling	RNC	%	Root Nitrogen Content			
	SRL	$m.g^{-1}$	Specific Root Length			
	nit	$mg.g^{-1}.L^{-1}.min^{-1}$	NO_3^-- absorption			
	amo	$mg.g^{-1}.L^{-1}.min^{-1}$	NH_4^+ absorption			
	DEA	$\mu gN\text{-}N_2O.g$ dry $soil^{-1}.h^{-1}$	Denitrification			
	NL1	%	Flag leaf nitrogen content	Flowering	Field conditions, 170 plants m^{-2}	Grignon, 2014
Architecture	RD	mm	Mean root diameter	8 weeks	Greenhouse conditions; 2L pots with sand and hydroponic solution, 3 plants per pot	Lyon, 2014
	RNb	#	Mean root number	6 weeks	Hydroponic growth in a 2D rhyzotron	Clermont, 2014
	RA	Degree	Mean root angle			
	L1MD	$g.cm^{-2}$	Flag leaf dry mass density	Flowering	Field conditions, 170 plants m^{-2}	Grignon, 2014
	S4L	cm^2	Surface of the four superior leaves			
	MSH	cm	Mean height of the main stem shoot	Grain filling	Field conditions, 100 plants m^{-2}	Le Moulon, 2014
Ground cover capacity	GAIT1	No dimension	Green Area Index in December (ratio of leaf green area to the area of ground)	Leaf 2 has emerged	Field conditions, 170 plants m^{-2}	Grignon, 2014
	GAIT6	No dimension	Green Area Index in April (ratio of leaf green area to the area of ground)	Stem elongation		
	Agg	No dimension	Capacity of compensation between two seeding densities (Ratio of the ear density between sowing at 36 and 170 plant m^{-2})	Grain filling	Field conditions, 36 and 170 plant m^{-2}	
	EarD	$Ears.m^{-2}$	Ear density per square meter		Field conditions, 150 plants m^{-2}	Le Moulon, 2014
	Agg2	No dimension	Aggressiveness index, computed as the ratio between tillering in low density, high nitrogen growing conditions (Grignon D2), and tillering in high density, low nitrogen (Le Moulon).		Field conditions, 150 plants m^{-2} (Grignon) and 170 plants m^{-2} (Le Moulon)	Le Moulon & Grignon, 2014
Disease	Yr	%	Sensitivity to yellow rust, percentage of the leaf surface attacked	Tillering to flowering	Compilation of data from ARVALIS, and the ECOGER and ECOSYS laboratories	
	Septo	%	Sensitivity to septoria, percentage of the leaf surface attacked			
Yield components	VEL	No dimension	Vertical coefficient of extinction of light	Stem elongation	Field conditions, 170 plants m^{-2}	Grignon, 2014
	EarP	$Ears/plant^{-1}$	Mean number of ears per plant		Field conditions, 100 plants m-2	Le Moulon, 2014
	FD	Days	Flowering date			
	TKW	g	Thousand Kernels Weight	Post Harvest		
	KEar	$Kernels.ear^{-1}$	Mean number of kernels per ear			

Table 3: Description of functional groups

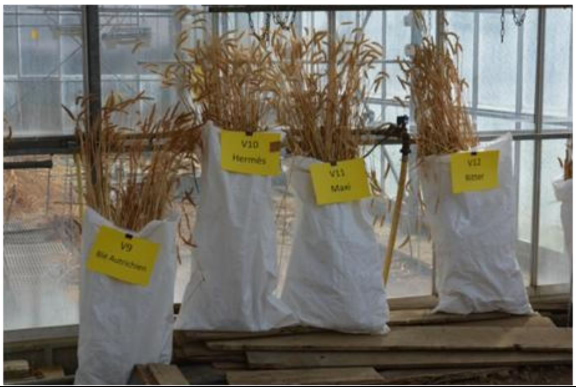
Variety name	**Functional groups**	**Description**
Altigo Trémie F426 A22	c1 	Varieties sensitive to fungal diseases (Septo, YR) and with weak flag leaf nitrogen content (NL1) **Functional group sensitive to fungal diseases**
Renan Skerzzo Midas Alauda	c2 	Varieties with short roots (SRL), high level of NO_3- absorption (nit) and high relative growth rate (RGR) **Functional group with limited soil exploration**
Blé Autrichien Hermès Maxi Ritter	c3 	Varieties with tall main stem (MSH), with high level of NH_4+ absorption (amo) and not very aggressive (Agg) **Functional group with slow phenology**
Grapeli Soissons Arezzo Boregar	c4 	Varieties with long roots (SRL), low level of NO_3^- absorption (nit) and low relative growth rate (RGR) **Functional group with good soil exploration**

Table 4: Synthetic view on the experimental plan used, with the list of all plots indicating for each plot their composition in term of variety number, number of variety groups varieties present (V1: Altigo, V2: Trémie, V3: F426, V4: A22, V5: Renan, V6: Skerzzo, V7: Midas, V8 Alauda, V9: Blé Autrichien, V10: Hermès, V11: Maxi, V12: Ritter, V13: Grapeli, V14: Soissons, V15: Arezzoet V16: Boregar). The number of replicate of each plot is also indicated.

				Functional groups															
				c1				c2				c3				c4			
Plot	# varieties	# groups	# replicates	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16
1	1	1	3	■															
2					■														
3						■													
4							■												
5								■											
6									■										
7										■									
8											■								
9												■							
10													■						
11														■					
12															■				
13																■			
14																	■		
15																		■	
16																			■
17	2	1	1	■	■														
18						■	■												
19								■	■										
20										■	■								
21												■	■						
22														■	■				
23																■	■		
24																		■	■
25		2	1	■				■											
26					■				■										
27						■								■					
28							■								■				
29				■												■			
30					■												■		
31										■				■					
32											■				■				
33								■								■			
34									■								■		
35												■						■	
36													■						■
37						■				■									
38							■					■							
39											■								■
40													■					■	
41	4	1	1	■	■	■	■												
42								■	■	■	■								
43												■	■	■	■				
44																■	■	■	■
45		2	1	■	■			■	■										
46						■	■							■	■				
47				■	■											■	■		
48						■	■											■	■
49								■	■			■	■						
50										■	■			■	■				
51										■	■							■	■
52												■	■			■	■		
53		3	1	■		■		■				■							

54			
55			
56			
57			
58			
59			
60			
61		4	1
62			
63			
64			
65			
66			
67			
68			
69	8	2	1
70			
71			
72			
73			
74			
75		3	1
76			
77			
78			
79			
80			
81			
82			
83		4	1
84			
85			
86			
87			
88			

Table 5: Number of plots for each combination of variety number and number of functional groups. Numbers in italic refer to replicates of monocultures already counted once in the plain figures.

		Number of functional groups				
		1	**2**	**3**	**4**	
Number of varieties	**1**	16x3				**16*+32***
	2	8	16			**24**
	4	4	8	8	8	**28**
	8		6	8	6	**20**
		28+ *32*	**30**	**16**	**14**	

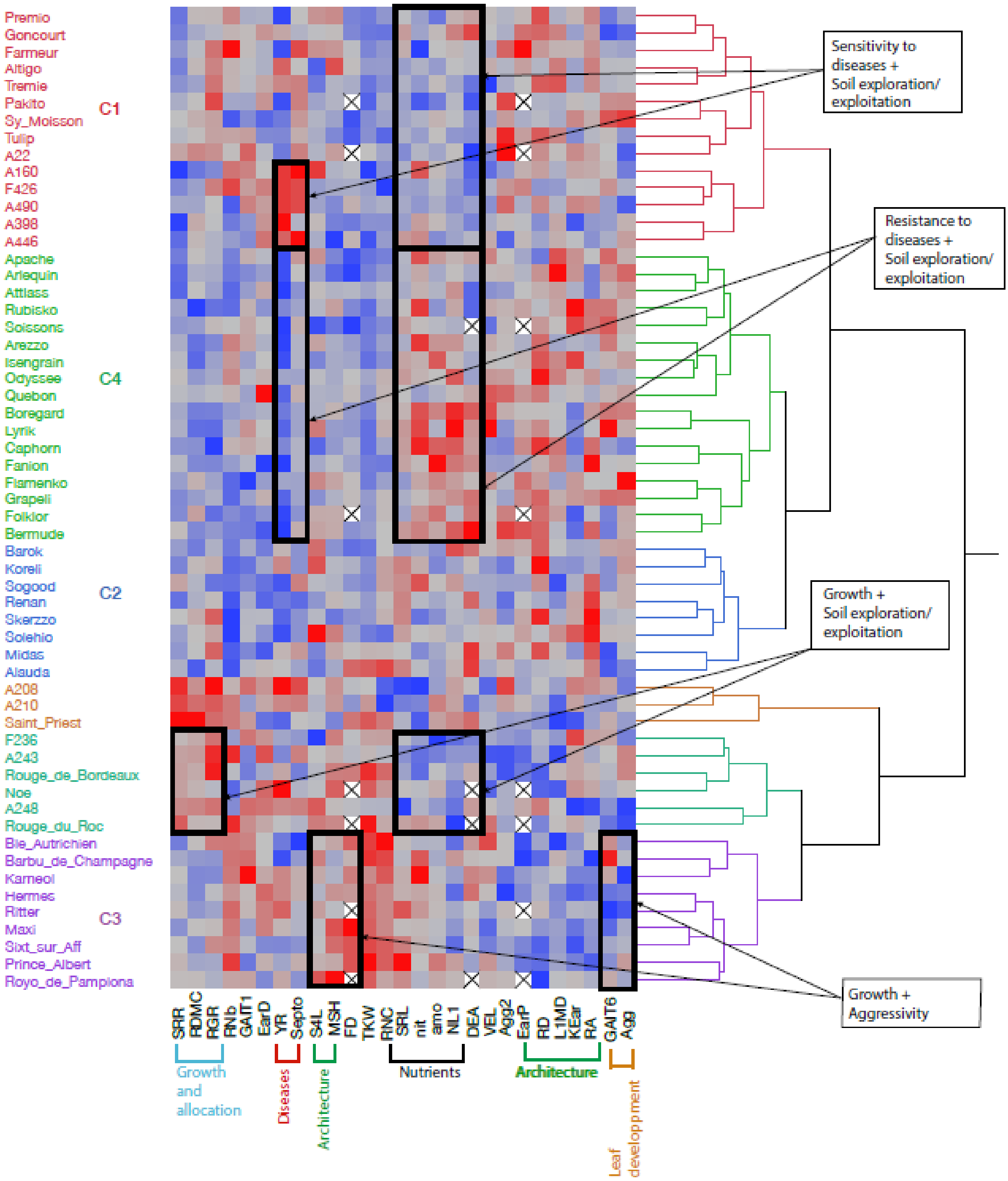


Fig. 1: Heatmap of the doubly ordered dendrograms done on wheat traits and lines. The shading from red to blue represents gradation from low to high trait values.

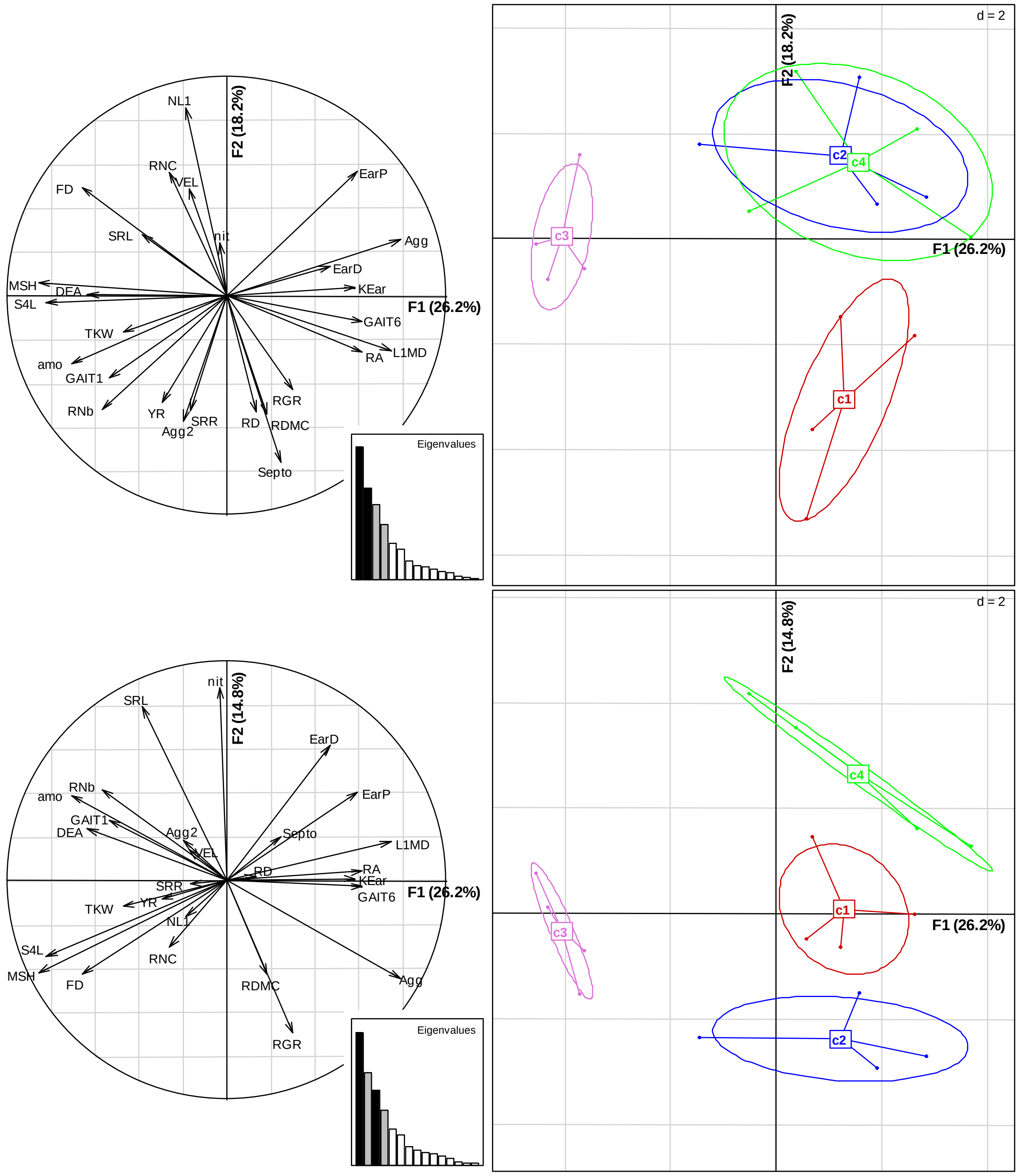


Fig. 2: Principal component analysis using traits of the 16 varieties (Table 2). Top left: Correlation circle plot of the first two principal components. Bottom left: Correlation circle plot of the first and third principal components. Vector labels correspond to trait codes in Table 2. Vector size is proportional to their contribution to axes. Top right: Projection of dataset variability plotted on a factorial map of the first two principal components. Bottom right: Projection of dataset variability plotted on a factorial map of the first and third principal components. Labels on the gravity centers correspond to functional groups (c1-c4). Eigen values 26.2, 18.2, 14.8% for axes 1 to 3, respectively.

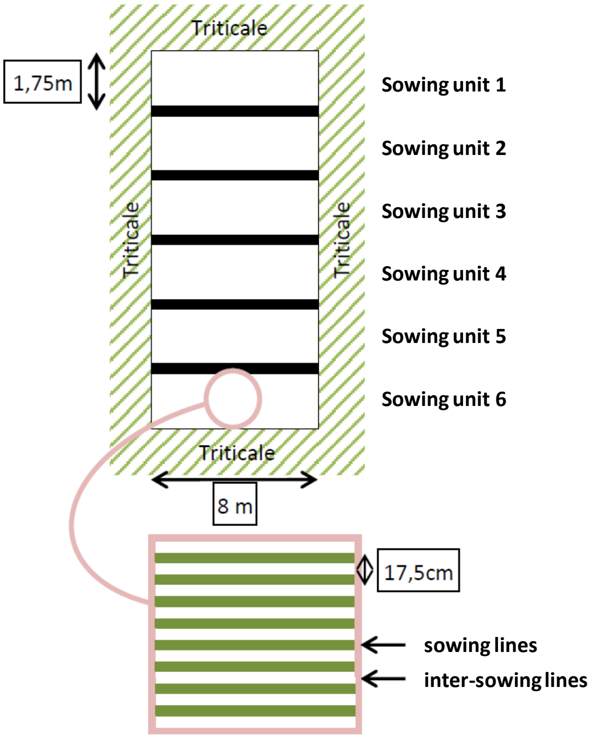


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Fig. 3: Experimental plot design.