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Essential oil chemotype diversity and environmental stability in Turkish basil (Ocimum basilicum L.) germplasm: a three-layer field evaluation across contrasting ecologies

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Abstract

The environmental stability of essential oil (EO) chemotype expression in basil (Ocimum basilicum L.) is critical for both industrial quality assurance and food safety, yet remains poorly characterised across the multiple dimensions of environmental variation encountered in production. This study characterises the environmental modulation of EO yield and chemotype composition in 12 basil genotypes representing four chemotypes (linalool, estragole, citral, and methyl cinnamate) using a three-layer experimental design: (i) ecological variation across three contrasting Turkish locations (Bursa, Eskişehir, Tokat) over two years, (ii) ontogenetic variation across three developmental stages at Bursa, and (iii) seasonal variation between summer and autumn harvests at Tokat (144 unique EO profiles). EO yield was highest at Bursa (0.98 mL·100 g^{-1} dry weight), increased from pre-flowering to onset of flowering, and was greater in autumn than summer harvests. The estragole chemotype (R-10A) exhibited exceptional compositional stability across ecological environments (Layer 1 CV = 2.8%), while the citral (Layer 1 CV = 12.9%) and methyl cinnamate (20.0%) chemotypes showed moderate to variable stability. Within the linalool chemotype, R-16 was the most stable genotype (SD = 5.6%). The results indicate that chemotype stability is primarily genotype-driven and that environment × harvest-stage interactions modulate secondary metabolite accumulation patterns in a chemotype-dependent manner, with implications for cultivar selection and food safety considerations.

Introduction

Sweet basil (Ocimum basilicum L., Lamiaceae) is one of the most commercially important aromatic herbs worldwide, valued for its culinary, medicinal, cosmetic, and industrial applications (Makri and Kintzios 2008; Dhama et al. 2021). The species exhibits remarkable intraspecific diversity in both morphological traits and secondary metabolite profiles (Labra et al. 2004; De Masi et al. 2006), and its botany, phytochemistry, and pharmacological properties have been comprehensively reviewed (Dhama et al. 2021; Azizah et al. 2023). Essential oil (EO) composition is the primary criterion for commercial classification of basil, as it determines both aroma characteristics and biological activity (Hussain et al. 2008).

Several chemotype classification systems have been proposed for O. basilicum based on the dominant EO constituents. Grayer et al. (1996) distinguished five major EO profiles based on the proportions of linalool, estragole, eugenol, methyl eugenol, and geraniol. Working with Turkish landraces, Telci et al. (2006) identified seven chemotypes from 18 accessions, expanding the known chemical diversity of Anatolian basil populations. Subsequent molecular and chemometric studies of Turkish germplasm confirmed this intraspecific diversity (Giachino et al. 2014). More recently, multivariate approaches have become standard for chemotype classification (Kirci et al. 2025), enabling finer resolution of compositional variation across germplasm collections. The chemodiversity of Ocimum species has been comprehensively reviewed by Gurav et al. (2022) and Dhama et al. (2021).

While the genetic component of EO composition is well established, the extent to which environmental factors modulate chemotype expression remains inadequately characterised, particularly in multi-location, multi-year field trials. The genotype × environment (G × E) interaction for EO traits has been documented in several aromatic species including Origanum vulgare (Lukas et al. 2015) and Salvia species (Ferrante et al. 2021), but comprehensive G × E studies in basil remain limited. Understanding EO chemotype stability is essential for growers, breeders, and the industry, ensuring reliable raw material quality (Pimentel et al. 2023).

Beyond spatial (ecological) variation, temporal factors also influence EO accumulation and composition. Ontogenetic variation in terpene and phenylpropanoid accumulation has been reported in basil (Eskandarzade et al. 2024; Beatović et al. 2015) and other Lamiaceae (Iijima et al. 2004a). EO yield generally increases from vegetative to flowering stages, while compound proportions may shift in parallel with changes in terpene synthase activity during glandular trichome maturation (Iijima et al. 2004a). Recent studies confirm that developmental stage is a critical determinant of EO quality at harvest (Kholiya et al. 2022; Mkaddem Mounira et al. 2022). In regions where multiple harvests per season are feasible, the harvest season can alter EO yield and composition due to changing photoperiod, temperature, and precipitation (Pimentel et al. 2023; Corrado et al. 2020).

A further practical dimension concerns food safety. Estragole (methyl chavicol) and methyl eugenol are phenylpropanoids classified as naturally occurring genotoxic and carcinogenic (in rodent models) substances by the European Medicines Agency (EMA 2023) and the Scientific Committee on Food (SCF 2001a, 2001b). Regulation (EC) No. 1334/2008 restricts these substances in foodstuffs and flavourings. Understanding how their proportions vary with genotype, location, and developmental stage is directly relevant to food safety risk assessment, particularly as basil is widely used as a fresh herb and flavouring agent in the food industry (Hamid et al. 2024; Hallmann and Ponder 2024). The authentication of basil products with respect to genotoxic phenylpropanoids has been recognised as an emerging food safety priority (Ríos-Rodríguez et al. 2021; Mahendran and Vimolmangkang 2023). EFSA opinions on estragole (EFSA CEF Panel 2012) and methyl eugenol (EFSA CEF Panel 2009) provide the regulatory genotoxicity framework, while basil matrix effects on estragole bioactivation have also been documented (Jeurissen et al. 2008).

Turkey harbours considerable basil genetic diversity in locally cultivated populations known as "reyhan" (Grayer et al. 1996). Despite this potential, Turkish basil remains insufficiently characterised regarding EO stability across environments. To the best of our knowledge, no study has simultaneously evaluated the ecological, ontogenetic, and seasonal variation of basil EO in a multi-year, multi-location design within Turkey.

The present study was designed to characterise the environmental modulation of EO yield and chemotype composition in 12 basil genotypes representing four chemotypes, using a three-layer experimental approach. Unlike standard genotype × environment interaction studies that examine EO variation across a single experimental dimension (typically location or harvest time), the three-layer

design simultaneously addresses ecological, ontogenetic and seasonal variation within the same genotype panel, enabling integrated cultivar recommendations that account for all three sources of compositional instability. The three layers are: (i) ecological variation across three locations; (ii) ontogenetic variation across three developmental stages; and (iii) seasonal variation between summer and autumn harvests (Fig. 1). The specific objectives were to quantify G × E interactions for EO yield and major compounds, to assess chemotype stability using CV analysis, and to evaluate the implications of compositional variation for food safety.

Materials and methods

Plant material

The plant material used in this study was derived from a basil germplasm collection established within a nationally funded research project (TÜBİTAK Project No. 111O677) based on local populations collected from different regions of Türkiye, together with a limited number of introduced genotypes. After preliminary characterisation of 70 accessions, 12 genotypes were selected based on prior chemotype characterisation (Kirci et al. 2025) to represent the four dominant chemotypes (linalool, estragole, citral, and methyl cinnamate) and a wide essential-oil yield range while ensuring suitability for multi-location field trials. Taxonomic identity as Ocimum basilicum L. was confirmed by Prof. Dr. İsa Telci, Department of Industrial Crops, Isparta University of Applied Sciences, Türkiye. The evaluated materials are maintained as documented accessions in the institutional medicinal and aromatic plants collection, and accession records are available for verification upon request. Accession origins, chemotype assignment and collection details are provided in Online Resource 1 (Table S2).

Experimental design and trial locations

Field trials were conducted at three ecologically contrasting locations in Turkey over two consecutive years (Year 1 and Year 2) (Online Resource 1, Table S3). The trial locations – Bursa (40°13'N, 28°51'E, 120 m a.s.l.), Eskişehir (39°45'N, 30°33'E, 789 m a.s.l.), and Tokat (40°19'N, 36°27'E, 594 m a.s.l.) – represent sub-Mediterranean, continental transition, and semi-arid continental climates, respectively. Growing-season (May–September) mean temperature, total precipitation, and mean relative humidity were 21.5°C, 198–425 mm, and 61–72% at Bursa; 18.1°C, 130–258 mm, and 55–67% at Eskişehir; and 18.5–20.9°C, 156–187 mm, and 50–55% at Tokat, respectively, across the two experimental years (Online Resource 1, Table S3). Soil at each site was classified as clay-loam (Bursa), silty-clay (Eskişehir), and sandy-loam (Tokat). Monthly climatic data are provided in Online Resource 1, Table S3.

Seedlings were produced from seed in perlite–peat mixture (5:1, v/v) and transplanted to each location after the last spring frost. The experiment was arranged in a randomised complete block design (RCBD) with three replications. Plant spacing was 40 × 30 cm. Prior to transplanting, 25 kg ha^{-1} pure nitrogen

(half of the total 50 kg ha^{-1}) and 50 kg ha^{-1} triple superphosphate were applied. Drip irrigation was provided as needed.

Three-layer experimental structure

Layer 1 – Ecological variation: All 12 genotypes were harvested at the onset of flowering at each of the three locations in both years, yielding 12 genotypes × 3 locations × 2 years = 72 EO profiles. Note: the onset-of-flowering harvest at Bursa in Layer 1 is shared with the onset-of-flowering (OF) stage in Layer 2, and the first harvest at Tokat in Layer 1 is shared with the summer harvest in Layer 3; the total number of unique EO profiles is 144, while the total number of layer-specific observations is 192. Because Layers 2 and 3 used subsets of the Layer 1 dataset, results across layers should be interpreted as complementary rather than statistically independent; ontogenetic conclusions are specific to Bursa and seasonal conclusions are specific to Tokat within the tested years.

Layer 2 – Ontogenetic variation: At the Bursa location, plant material was sampled at three developmental stages in both years: (i) pre-flowering (PF, vegetative), (ii) onset of flowering (OF, 10–15% open flowers), and (iii) full flowering (FF, > 50% open flowers), yielding 72 EO profiles.

Layer 3 – Seasonal variation: At the Tokat location, two harvests were taken per growing season in both years: a summer harvest (first cut, June–July) and an autumn harvest (second cut, August–September), yielding 48 EO profiles.

Essential oil extraction

EO was extracted from dried leaf material, consistent with established practice in multi-genotype basil EO evaluation where simultaneous processing of numerous samples precludes immediate distillation of fresh tissue (Beatović et al. 2015; Kholiya et al. 2022). Leaf samples were dried in a forced-air oven at 35°C to constant weight (approximately 5–7 days), protected from direct light; this low-temperature drying protocol minimises loss of volatile monoterpenes while enabling standardised sample preparation across locations and harvest dates. Dried leaves were stored in sealed dark-glass containers at ambient temperature until distillation, within two weeks of drying to minimise monoterpene loss. Fifteen grams of dried leaf were placed in a round-bottom flask with 300 mL distilled water (1:20, w/v) and subjected to hydrodistillation for 2 h using a Neo-Clevenger-type apparatus following the European Pharmacopoeia protocol (European Pharmacopoeia Commission 2010). EO yield was expressed as mL·100 g^{-1} dry weight (DW), numerically equivalent to % v/w. EO volume was read at ambient temperature after complete phase separation in the graduated Clevenger tube.

Gas chromatography and gas chromatography–mass spectrometry

GC analysis was performed on a Perkin-Elmer (PerkinElmer, Waltham, MA, USA) gas chromatograph equipped with an autosampler and flame ionisation detector (FID). Samples were diluted 1:10 in acetone and separated on a BPX5 capillary column (SGE Analytical Science, Melbourne, Australia; 30 m × 0.25 mm i.d. × 0.25 µm). Peaks were integrated using PerkinElmer TotalChrom software with automatic baseline detection and manual correction where necessary; integration settings were applied uniformly across all samples. Helium carrier gas at 5 psi; injector 230°C; detector 250°C; oven programme: initial 60°C (hold 2 min), ramped at 3°C·min⁻¹ to 230°C (hold 15 min); total run time 73.7 min; split injection 5:1. Quantitative data are expressed as relative FID peak area (%). Response factor correction was not applied; accordingly, all compositional values represent relative proportions rather than absolute mass concentrations. For the dominant compounds verified against authentic standards (linalool, estragole, methyl eugenol, methyl cinnamate, eugenol, geraniol), the relative FID response approximates mass proportions within ± 5% for the monoterpene and phenylpropanoid classes studied (Amor et al. 2021; Šovljanski et al. 2022). This approach is standard in multi-genotype EO characterisation (Amor et al. 2021; Šovljanski et al. 2022; Gurkan and Hayaloglu 2023; Abdoul-Latif et al. 2022). The BPX5 column was selected for broad-spectrum separation across four diverse chemotypes; while it has limited selectivity for co-eluting phenylpropanoid isomers, this does not affect the findings given the unambiguous dominance of identified compounds in each chemotype. Co-elution of estragole with trans-anethole was checked by RI comparison (estragole RI = 1195 vs. trans-anethole RI = 1283 on BPX5) and confirmed absent in all samples. The FID quantification approach, applied consistently across all 144 unique EO profiles in the present study, does not affect between-genotype or between-environment comparisons.

Gas chromatography–mass spectrometry (GC-MS) analysis used a PerkinElmer GC-MS system (PerkinElmer, Waltham, MA, USA) (electron ionisation at 70 eV), same column and temperature programme, helium at 1.3 mL·min⁻¹, transfer line 250°C. Compound identification was based on: (i) comparison with authentic reference standards (linalool, estragole, methyl eugenol, methyl cinnamate, eugenol, geraniol, and α-terpineol; Sigma-Aldrich, St. Louis, MO, USA; ≥98% purity); (ii) retention index (RI) calculation relative to a homologous n-alkane series (C_8–C_{20}); and (iii) mass spectral matching against Wiley and NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral databases (minimum forward match score ≥ 850/1000) and published data (Adams 2017). Compounds present at less than 0.1% relative peak area were not included in quantitative reporting; this threshold corresponds to approximately 3× the mean injection-to-injection variability (RSD < 3%), ensuring only reliably quantifiable peaks are reported. Retention indices (RI) for all identified compounds are provided in Online Resource 1 (Table S4). Identification was assigned based on agreement between RI (typically within ± 10 units) and MS library matching; dominant compounds were additionally confirmed using authentic standards where available (Adams 2017; Jiang et al. 2024).

Statistical analysis

Sampling and analytical replication. At each harvest, 10–15 plants were sampled randomly from each replication block. Leaf material from each replicate block was pooled, mixed, and a 15 g subsample was

taken for hydrodistillation. This pooling eliminates within-plot (plant-to-plant) variation; genotypes with heterogeneous individual-plant EO profiles would appear artificially stable from pooled samples. Consequently, the CV-based stability estimates reported here reflect between-environment variation only and may underestimate true total variation, which would include the within-plot component. Each replicate was distilled and analysed separately, yielding three independent analytical observations per genotype per environment. GC-FID analysis involved a single injection per sample; peak area reproducibility was confirmed by duplicate injection of a random subset (n = 12 samples), with a mean relative standard deviation below 3%. Mean ± standard deviation (SD) values reported in tables and figures are based on these three replicate observations within each environment–year combination, except where stated otherwise. Sample processing (drying, distillation, and GC analysis) was randomised across locations and years within each analytical batch to minimise systematic confounding of batch effects with environmental effects.

Analysis of variance (ANOVA) was performed separately for each experimental layer. In Layer 1, genotype (G), location (L) and year (Yr) were included as fixed factors, and all two- and three-way interactions (G×L, G×Yr, L×Yr and G×L×Yr) were tested. Replications were nested within each location × year combination. Year was treated as a fixed effect because the two experimental years represent the specific study period rather than a random sample; thus, inference applies to Year 1–Year 2 conditions (Online Resource 1, Table S3), and extrapolation to other years should be made cautiously. Analogous models were fitted for Layers 2 and 3. Mean separation was performed using Duncan's multiple range test at $P \leq 0.01$ (Eberhart and Russell 1966). Key conclusions were verified using Tukey's HSD ($\alpha = 0.05$) with consistent outcomes (Online Resource 1, Table S1).

EO composition data are presented as relative FID peak area proportions (%, normalised to total detected peak area). Because inference focused on single dominant compounds constituting more than 30% of total EO (linalool, estragole, citral, or methyl cinnamate), the summation constraint has minimal distorting effect on individual variances. Analyses were nevertheless repeated after arcsine square-root transformation; rankings and significance conclusions were unchanged. Untransformed values are therefore reported throughout for ease of interpretation, consistent with established practice in multi-genotype basil EO studies.

Duncan's multiple range test was selected because the primary objective was genotypic discrimination within the fixed set of 12 tested genotypes, rather than control of familywise error across a large set of random comparisons. This choice is consistent with established practice in aromatic crop genotype evaluation. As a robustness check, all pairwise conclusions (e.g., Bursa vs. Eskişehir and Tokat for EO yield; R-10A estragole stability vs. R-17 citral; R-16 and R-20 linalool stability rankings) were verified using Tukey's HSD at $P \leq 0.01$ and remained unchanged (i.e., no genotype pair that was significantly different under Duncan became non-significant under Tukey, confirming that the less conservative Duncan approach did not inflate Type I errors in this dataset). All analyses were performed using SAS 9.4 (PROC GLM with MEANS statement; SAS Institute, Cary, NC, USA). ANOVA F-ratios, degrees of freedom, and

probability values for main effects and interactions across all three experimental layers are provided as Online Resource 1 (Table S1).

Chemotype stability was quantified by the CV (%) of the dominant compound across three locations and two years (Layer 1 – ecological variation; n = 6 environment–year combinations per genotype), following the multi-environment crop evaluation convention (Zobel et al. 1988): CV < 10% = highly stable; 10–20% = moderately stable; CV ≥ 20% = variable.

Results

Essential oil yield

Ecological variation (Layer 1)

EO yield varied significantly ($P \leq 0.01$) among genotypes, locations, and years, and the genotype × location interaction was significant ($P \leq 0.01$), indicating differential genotypic responses to environmental conditions. The three-way G×L×Yr interaction was also significant ($P \leq 0.05$), though with modest statistical power (df = 22, 120); accordingly, interpretation focuses on main effects and two-way interactions (Table 1; Fig. 2). Across all genotypes and years, Bursa produced the highest mean EO yield (0.98 mL·100 g^{-1} DW), followed by Eskişehir (0.74) and Tokat (0.72). Year 2 produced significantly higher yields than Year 1 (0.88 vs. 0.75 mL·100 g^{-1} DW; $P \leq 0.01$).

Table 1
Essential oil (EO) yield (mL·100 g⁻¹ dry weight) of 12 basil genotypes across three locations over two years (Layer 1 - ecological variation).

Genotype	Bursa Year 1	Bursa Year 2	Eskişehir Year 1	Eskişehir Year 2	Tokat Year 1	Tokat Year 2	Mean
R-1	0.89	0.82	0.6	0.88	0.51	0.62	0.72 efg
R-3k	0.67	1.37	0.82	0.73	0.66	1.11	0.89 d
R-4	0.53	0.86	0.66	0.67	0.69	0.62	0.67 fg
R-10A	1.58	1.61	1.13	1.04	1.03	1.16	1.26 b
R-15	1.1	1.58	1.22	0.95	0.82	1.13	1.13 bc
R-16	0.67	1.08	0.55	0.47	0.36	0.53	0.61 gh
R-17	0.87	1.1	0.82	0.87	0.82	0.82	0.88 d
R-19	1.11	1.01	0.82	0.8	0.69	0.73	0.86 de
R-20	0.58	0.67	0.54	0.43	0.36	0.47	0.51 h
R-23	1.29	1.71	1.23	1.17	0.91	1.67	1.33 a
Y-7	0.42	0.49	0.22	0.36	0.18	0.42	0.35 i
Y-15	0.73	0.7	0.32	0.4	0.44	0.51	0.52 h

Among genotypes, R-23 had the highest two-year mean EO yield (1.33 mL·100 g⁻¹ DW), followed by R-10A (1.26) and R-15 (1.13), while Y-7 had the lowest (0.35). The genotype × location interaction was particularly notable for R-10A, R-15, and R-23, which showed markedly higher yields at Bursa. Genotype R-4 maintained relatively stable yields across all three sites (range: 0.53–0.86 mL·100 g⁻¹ DW), suggesting high environmental stability for yield. These findings are consistent with multi-location yield stability analyses reported by Kholiya et al. (2022) and Tursun and Telci (2020).

Ontogenetic variation (Layer 2)

Developmental stage had a significant effect ($P \leq 0.01$) on EO yield (Table 2; Fig. 3). Pre-flowering plants produced the lowest mean yield (0.78 mL·100 g⁻¹ DW), while onset of flowering and full-flowering stages produced similar and significantly higher values (0.98 and 1.00, respectively). Most genotypes followed the general trend of increasing EO yield with advancing maturity, consistent with the role of glandular trichome development in EO accumulation (Gang et al. 2001), and aligning with recent studies by Mkaddem Mounira et al. (2022) and Corrado et al. (2020). However, R-10A reached its maximum yield at onset of flowering rather than full flowering (1.58–1.61 vs. 1.38–1.51 mL·100 g⁻¹ DW in Year 1 and Year

2, respectively), which may reflect the earlier peak accumulation of phenylpropanoid-dominated EOs reported in basil (Iijima et al. 2004a).

Table 2
Essential oil (EO) yield (mL·100 g⁻¹ dry weight) of 12 basil genotypes across three developmental stages at Bursa (Layer 2 - ontogenetic variation).

Genotype	PF Year 1	PF Year 2	OF Year 1	OF Year 2	FF Year 1	FF Year 2	CV (%)	Stage mean
R-1	0.69	0.73	0.89	0.82	1.02	0.77	14.7	0.82
R-3k	0.93	0.83	0.67	1.37	1	1.04	24.2	0.97
R-4	0.78	0.61	0.53	0.86	0.96	0.69	21.7	0.74
R-10A	1.02	1.27	1.58	1.61	1.51	1.38	16.0	1.40
R-15	1.32	1.2	1.1	1.58	1.71	1.61	17.4	1.42
R-16	0.74	0.49	0.67	1.08	0.73	0.76	25.7	0.74
R-17	1.02	0.53	0.87	1.1	0.94	1.09	23.0	0.92
R-19	0.94	0.67	1.11	1.01	1.13	0.73	20.7	0.93
R-20	0.44	0.53	0.58	0.67	0.69	0.57	15.9	0.58
R-23	1.18	1.01	1.29	1.71	1.53	1.76	21.3	1.41
Y-7	0.29	0.37	0.42	0.49	0.4	0.49	18.6	0.41
Y-15	0.64	0.37	0.73	0.7	1	0.38	37.3	0.64

Seasonal variation (Layer 3)

Autumn harvests produced significantly higher EO yields than summer harvests in both years (0.72 vs. 0.58 mL·100 g⁻¹ DW; $P \leq 0.01$; Table 3). The year × season interaction was significant, with the difference more pronounced in Year 2 (0.82 vs. 0.58) than in Year 1 (0.62 vs. 0.57). All genotypes produced higher EO yields in autumn, supporting the value of double-cropping strategies reported by Corrado et al. (2020) and Ciriello et al. (2021). R-23 showed the largest seasonal difference, while R-17 was relatively stable across seasons.

Table 3
Essential oil (EO) yield (mL·100 g⁻¹ dry weight) of 12 basil genotypes across two harvest seasons at Tokat (Layer 3 - seasonal variation).

Genotype	Summer Year 1	Summer Year 2	Autumn Year 1	Autumn Year 2	Summer mean	Autumn mean	CV (%)
R-1	0.56	0.49	0.51	0.62	0.53	0.56	10.6
R-3k	0.47	0.69	0.66	1.11	0.58	0.89	36.8
R-4	0.47	0.6	0.69	0.62	0.53	0.66	15.4
R-10A	1.02	1.02	1.03	1.16	1.02	1.09	6.5
R-15	0.78	0.73	0.82	1.13	0.76	0.97	20.9
R-16	0.36	0.36	0.37	0.53	0.36	0.45	20.6
R-17	0.82	0.8	0.82	0.82	0.81	0.82	1.2
R-19	0.62	0.49	0.69	0.73	0.55	0.71	16.7
R-20	0.2	0.33	0.36	0.47	0.27	0.41	32.7
R-23	0.96	0.76	0.91	1.67	0.86	1.29	37.7
Y-7	0.14	0.36	0.18	0.42	0.25	0.30	49.5
Y-15	0.49	0.31	0.44	0.51	0.40	0.47	20.6

Essential oil composition

Chemotype overview

GC-MS analysis confirmed four distinct chemotypes in the evaluated genotypes, with Layer 1 compositional summaries presented in Table 4: (i) linalool chemotype (nine genotypes: R-1, R-3k, R-4, R-15, R-16, R-19, R-20, Y-7, Y-15; note: R-4 is classified as linalool-type based on its dominant compound but uniquely co-accumulates methyl eugenol at 9.33%) with linalool ranging from 51.7 to 71.2% (grand means across six environment–year combinations; Table 4); (ii) estragole chemotype (R-10A) with estragole at 93.5%; (iii) citral chemotype (R-17; citral reported as the sum of its geometric isomers neral [citral b] and geranial [citral a]) with citral (geranial + neral) at 75%; and (iv) methyl cinnamate chemotype (R-23) with methyl cinnamate at 43.1% and linalool as the major secondary constituent at 34.9%. Other notable secondary compounds included eugenol (up to 13.7% in Y-7), α-bergamotene (up to 8.37% in R-19), and δ-cadinene (up to 8.45% in R-20). This chemotypic diversity mirrors the broad chemical variation documented by Gurkan and Hayaloglu (2023) and Bajomo et al. (2022) for commercially available basil varieties, and is consistent with the volatile profiles reported by Qasem et al. (2023).

Table 4
Major essential oil (EO) compound relative proportion (%, uncorrected FID peak area) in basil genotypes across three locations and two years (mean ± standard deviation (SD) across six environment-year combinations; Layer 1 - ecological variation).

Chemotype	Genotype	Bursa Year 1	Eskişehir Year 1	Tokat Year 1	Bursa Year 2	Eskişehir Year 2	Tokat Year 2	Mean ± SD
Linalool	*R-1*	49.07	59.56	77.83	56.77	56.03	56.30	59.30 ± 9.7
	R-3k	62.34	66.40	80.93	54.03	56.59	66.28	64.40 ± 9.5
	R-4	50.53	34.49	88.43	48.85	53.57	55.09	55.20 ± 17.9
	R-15	35.51	63.43	85.34	67.96	66.51	62.24	63.50 ± 16.1
	R-16	66.21	72.86	79.89	64.54	69.75	73.72	71.20 ± 5.6
	R-19	50.95	53.88	73.66	47.81	51.65	55.16	55.50 ± 9.2
	R-20	75.79	69.76	82.96	62.13	59.45	73.54	70.60 ± 8.8
	Y-7	43.91	68.90	60.85	44.18	44.90	50.12	52.10 ± 10.4
	Y-15	50.55	53.79	61.81	50.87	46.97	46.22	51.70 ± 5.7
Estragole	*R-10A*	91.71	94.94	98.13	92.56	92.21	91.34	93.50 ± 2.6
Citral	*R-17*	77.31	74.42	89.54	70.57	60.12	77.77	75.00 ± 9.7
Methyl cinnamate	*R-23*	40.45	51.96	31.31	35.45	49.55	50.05	43.10 ± 8.6
Methyl eugenol	*R-4†*	8.97	9.14	9.52	9.67	9.28	9.41	9.33 ± 0.26

Linalool chemotype – environmental variation

Linalool content in the nine linalool-type genotypes varied considerably across environments (Fig. 5). In Layer 1, the mean linalool content was highest at Tokat (Year 1: 76.86%, Year 2: 59.85%), followed by Eskişehir and Bursa. This inverse relationship between EO yield and linalool proportion may reflect a dilution effect, wherein higher total EO production at warmer sites is accompanied by proportionally greater accumulation of minor terpenes (Marotti et al. 1996), consistent with findings of Tursun and Telci (2020).

Individual genotypes differed markedly in linalool stability. R-16 and R-20 maintained relatively high and stable linalool proportions across all three locations (R-16: 64.54–79.89%; R-20: 59.45–82.96%). In contrast, R-4 showed the greatest location-dependent variation (34.49–88.43%), indicating high G × E interaction for this trait. In Layer 2 (ontogenetic variation), linalool content showed a general decline from pre-flowering to full flowering (Year 1 means: 57.23%, 55.38%, 52.06%), consistent with findings of Eskandarzade et al. (2024) and Beatović et al. (2015). The phenolic composition and maturity interactions reported by Lawson et al. (2025) further support the role of developmental stage in secondary metabolite accumulation. In Layer 3 (seasonal), linalool content was generally similar between summer and autumn harvests (Fig. 4), suggesting strong genetic control over linalool biosynthesis.

Estragole chemotype – environmental stability

Genotype R-10A maintained remarkably high and stable estragole proportions across all experimental layers (Fig. 6a). In Layer 1, estragole ranged from 91.34 to 98.13% (Year 1 vs. Year 2 values shown in Table 4). In Layer 2, estragole ranged from 86.8 to 94.47%. In Layer 3, estragole ranged from 88.62 to 98.72%. The Layer 1 CV was 2.8%, confirming exceptional compositional stability consistent with the strong genetic determination of the estragole chemotype reported by Telci et al. (2006) and corroborated by recent chemotyping studies (Kirci et al. 2025; Bajomo et al. 2022).

Citral chemotype – environmental sensitivity

The citral chemotype (R-17) showed substantial sensitivity across experimental layers, particularly in ontogenetic responses (Fig. 6b), although its Layer 1 CV (12.9%) indicated only moderate ecological instability. Citral content ranged from 60.12 to 89.54% in Layer 1 and from 37.17 to 78.16% in Layer 2, with dramatic year-to-year fluctuation in the pre-flowering stage (Year 1: 78.16% vs. Year 2: 37.17%). The Layer 1 CV was 12.9% (moderately stable), although inclusion of ontogenetic data substantially increased overall variability. This instability may be related to the environmental sensitivity of geraniol dehydrogenase, which catalyses the oxidation of geraniol to citral (Iijima et al. 2004b), and is consistent with the broad compositional variation observed for citral-type basil accessions by Avetisyan et al. (2017).

The methyl cinnamate chemotype (R-23) exhibited variable stability at the classification threshold (Layer 1 CV = 20.0%; Fig. 6c; Table 5), consistent with environmental sensitivity of methyl cinnamate accumulation in Lamiaceae (Telci et al. 2006).

Table 5

Chemotype stability assessment based on the coefficient of variation (CV%) of the dominant compound in Layer 1 (ecological variation; three locations × two years) for basil genotypes studied. n = number of genotypes per chemotype. CV (%) calculated across three locations and two years (Layer 1 – ecological variation; n = 6 environment–year combinations per genotype). Stability thresholds following multi-environment crop evaluation convention (Zobel et al. 1988): CV < 10% = highly stable; 10–20% = moderately stable; CV ≥ 20% = variable. Note: CV estimates are based on n = 6 environment-year means per genotype; confidence intervals may be wide for very low CV values.

Chemotype	Dominant compound	n	CV% range	Mean CV%	Stability class
Estragole	Estragole	1	2.8	2.8	Highly stable (CV < 10%)
Linalool	Linalool	9	7.8–32.4	17.4	Moderate to variable
Methyl cinnamate	Methyl cinnamate	1	20.0	20.0	Variable (CV ≥ 20%)
Citral	Citral (geranial + neral)	1	12.9	12.9	Moderate (10–20%)

Discussion

Ecological drivers of essential oil yield

The superiority of Bursa for EO yield may be partly attributed to its warmer and more humid microclimate. Growing-season mean temperature was 21.5°C at Bursa versus 18.1°C at Eskişehir and 19.7°C (two-year mean) at Tokat; relative humidity averaged 66.5% at Bursa, 61.1% at Eskişehir, and 52.2% at Tokat (Online Resource 1, Table S3), though other site-specific factors – including the well-structured clay-loam soils characteristic of the Bursa plain, longer effective sunshine duration at lower altitude (120 m), and irrigation management – cannot be excluded. Eskişehir (789 m altitude; drier, continental) and Tokat (594 m; semi-arid) represent substantially different growing conditions, and the altitude gradient of 669 m between the lowest (Bursa) and highest (Eskişehir) site likely contributes to the observed EO yield gradient through temperature and growing season effects. Temperature is a primary driver of EO biosynthesis in Lamiaceae, as monoterpene and sesquiterpene synthase activities are known to be temperature-dependent (Sangwan et al. 2001). It should be noted that drip irrigation was applied as needed without standardised protocols across locations; differential water supply may have contributed to the observed location effects alongside climatic differences. Hussain et al. (2008) reported similar positive correlations between environmental temperature and basil EO yield. Tursun and Telci (2020) further demonstrated that elevated CO_2 and temperature significantly modulate EO composition in purple basil. The significant genotype × location interaction confirms that cultivar selection must be location-specific for optimal EO production, as also emphasised by Rahimi et al. (2023) and Mulugeta et al. (2023) in the context of environmental stress responses. The comprehensive review by da Silva et al. (2021) further contextualises how extraction method and environmental conditions jointly determine basil EO yield and composition in food applications.

The between-year variation in EO yield (Year 2 > Year 1) across all locations underscores the importance of multi-year trials for reliable cultivar recommendations. Because Year was treated as a fixed effect, the conclusions strictly apply to the conditions tested and should not be generalised beyond the two-year window without further validation. Single-year data may substantially misrepresent a genotype's productive potential, a limitation commonly encountered in aromatic crop evaluation (Tena et al. 2019). The consistent year effect further supports treating year as a design factor rather than a random nuisance term in the present fixed-effects model.

Ontogenetic patterns and harvest timing

The 0.20 mL·100 g^{-1} DW increase in EO yield from the vegetative (0.78) to the onset of flowering stage (0.98), without a further significant increase at full flowering, provides clear harvest timing guidance. This pattern is consistent with the developmental trajectory of glandular trichomes as the primary site of EO biosynthesis and storage (Gang et al. 2001). Beatović et al. (2015) and Eskandarzade et al. (2024) reported similar ontogenetic patterns in Serbian and Iranian basil cultivars. The ontogenetic decline in linalool content, combined with no significant additional yield gain at full flowering, supports harvesting at onset of flowering to maximise both EO yield and linalool quality. This recommendation aligns with recent findings by Kholiya et al. (2022) and Mkaddem Mounira et al. (2022). For the estragole chemotype, the earlier yield peak at onset of flowering provides a practical distinction for production scheduling.

Seasonal variation and double-cropping potential

The significantly higher EO yield in autumn harvests compared to summer harvests at Tokat supports the agronomic viability of double-cropping strategies. Corrado et al. (2020) reported analogous harvest sequence effects on basil metabolic profiles, noting that successive harvests modify both yield and quality. Ciriello et al. (2021) demonstrated that the interaction between genotype and harvest sequence significantly affects phenolic acid and aroma profiles in Genovese basil, highlighting the need to optimise genotype–harvest scheduling combinations. The relatively stable EO composition across seasons in linalool-type genotypes suggests that autumn double-cropping can deliver comparable quality to the primary summer harvest, a commercially relevant finding for the basil food industry (Hamid et al. 2024; Hallmann and Ponder 2024).

Chemotype stability and implications for secondary metabolite management

The three-layer experimental design employed here provides a more comprehensive picture of chemotype stability than conventional single-dimension G×E studies, which typically assess only spatial variation. By simultaneously examining ecological, ontogenetic and seasonal effects, the present study reveals that compositional stability varies not only among genotypes but also among the dimensions of environmental variation tested. The CV-based stability assessment reveals a clear hierarchy of environmental sensitivity among basil chemotypes. The estragole chemotype (Layer 1 CV = 2.8%)

exhibited near-absolute compositional stability, suggesting strong genetic canalization of phenylpropanoid biosynthesis with minimal environmental modulation. This aligns with Telci et al. (2006), who noted the consistency of estragole-dominant chemotypes across Turkish growing conditions, and with recent chemotyping studies (Kirci et al. 2025; Bajomo et al. 2022). In contrast, the citral chemotype (Layer 1 CV = 12.9%) showed substantial environmental sensitivity, particularly across ontogenetic stages, consistent with the known variability of citral accumulation (Iijima et al. 2004b) and the instability of geraniol-derived compounds under fluctuating temperature and moisture regimes (Tursun and Telci 2020). It should be noted that the estragole, citral, and methyl cinnamate chemotypes are each represented by a single genotype (R-10A, R-17, and R-23, respectively); consequently, the stability values reported for these chemotypes reflect individual genotypic stability rather than chemotype-level generalisations. Additional genotypes within each non-linalool chemotype would be needed to confirm whether the observed stability patterns are chemotype-intrinsic.

Within the linalool chemotype, R-16 (SD = 5.6%) emerged as the only highly stable linalool genotype, while R-20 (SD = 8.8%) also showed comparatively low variability, together representing the most stable pair within the linalool chemotype. In contrast, R-4 (SD = 17.9%) showed the greatest compositional instability for linalool despite stable EO yield, confirming that yield stability and compositional stability are not necessarily correlated. The SD values for linalool genotypes are reported in absolute percentage points (rather than CV%) because the mean linalool content varies substantially across genotypes, making CV-based comparison misleading. It should be noted that all conclusions drawn regarding environmental (in)stability apply within the specific set of three Turkish locations, two growing years, and management conditions tested in the present study. It is acknowledged that quantitative EO data were obtained without FID response factor correction, representing relative rather than absolute proportions. This approach is widely accepted in multi-genotype EO characterisation studies (Šovljanski et al. 2022; Abdoul-Latif et al. 2022). For the dominant compounds verified against authentic standards, the relative FID response is expected to approximate mass proportions within about ± 5% for the monoterpene and phenylpropanoid classes studied.

It is acknowledged that CV-based stability, while practical and easily interpretable, has inherent limitations: it is sensitive to the mean value (genotypes with low mean proportions tend to have inflated CV%) and does not capture the pattern of instability (e.g., consistent direction vs. unpredictable fluctuation). Although the CV reported in Table 5 is based on Layer 1 (ecological variation) alone, the three locations span approximately 8° of longitude and 670 m of altitude; the inclusion of ontogenetic and seasonal layers in the broader study design shows that additional variance sources exist beyond the ecological dimension captured by CV. These considerations should be borne in mind when extrapolating the stability rankings to production environments outside the tested range.

Food safety implications of estragole and methyl eugenol variation

The margin of exposure (MOE) calculations presented here are illustrative only and are not intended for formal risk assessment because several assumptions were not experimentally determined (including the fresh-to-dry weight ratio). Using a nominal 5:1 fresh-to-dry ratio and typical fresh basil intake of 1–2 g per eating occasion, estimated MOEs for estragole in the estragole chemotype were generally above 10,000, whereas methyl eugenol in R-4 yielded lower MOEs. A high-consumer scenario (e.g., 10 g fresh basil per occasion) would substantially reduce MOEs; therefore, chemotype-based cultivar selection remains the most practical risk-management strategy. Regulation (EC) No. 1334/2008 maximum levels apply to estragole in the final food product as consumed, not to the raw herb or essential oil.

Given the genotoxic and carcinogenic classification of these phenylpropanoids, the MOE framework should be interpreted cautiously and chemotype-based cultivar selection is recommended.

Conclusions

This three-layer field study – encompassing 12 genotypes, three Turkish locations, three developmental stages, two harvest seasons, and two years (144 unique EO profiles; 192 layer-specific observations) – provides the first comprehensive characterisation of environmental, ontogenetic, and seasonal modulation of essential oil chemotype expression in basil. Key findings are: (i) Bursa optimises EO yield (0.98 mL·100 g^{-1} DW) while Tokat maximises linalool proportion; (ii) onset of flowering is the optimal harvest stage for both yield and quality; (iii) autumn harvests support double-cropping with comparable linalool quality; (iv) the estragole chemotype is exceptionally stable across ecological environments (Layer 1 CV = 2.8%) while the citral (12.9%) and methyl cinnamate (20.0%) chemotypes show moderate to variable stability; (v) R-16 is the most stable linalool genotype (SD = 5.6%). These results indicate that environment × harvest-stage interactions modulate secondary metabolite accumulation in a chemotype-dependent manner and that high-phenylpropanoid chemotypes (e.g., estragole and methyl eugenol) are primarily genotype-driven and should be managed through chemotype-based cultivar selection to support food safety-oriented cultivar deployment under EU flavouring regulations.

Declarations

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

Adem Zorlu: Conceptualization; Methodology; Investigation; Data curation; Formal analysis; Writing – original draft. İsa Telci: Conceptualization; Supervision; Methodology; Validation; Writing – review & editing. Mahfuz Elmastaş: Chemical analysis (GC–MS/GC–FID); Methodology; Validation; Data interpretation; Writing – review & editing. Oya Kaçar: Field trials and agronomic management (Bursa); Sampling; Resources; Writing – review & editing. Zehra Aytaç: Field trials and agronomic management

(Eskişehir); Sampling; Resources; Writing – review & editing. Emine Bayram: Field trial support and resources; Supervision; Writing – review & editing. All authors have read and approved the final version of the manuscript.

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

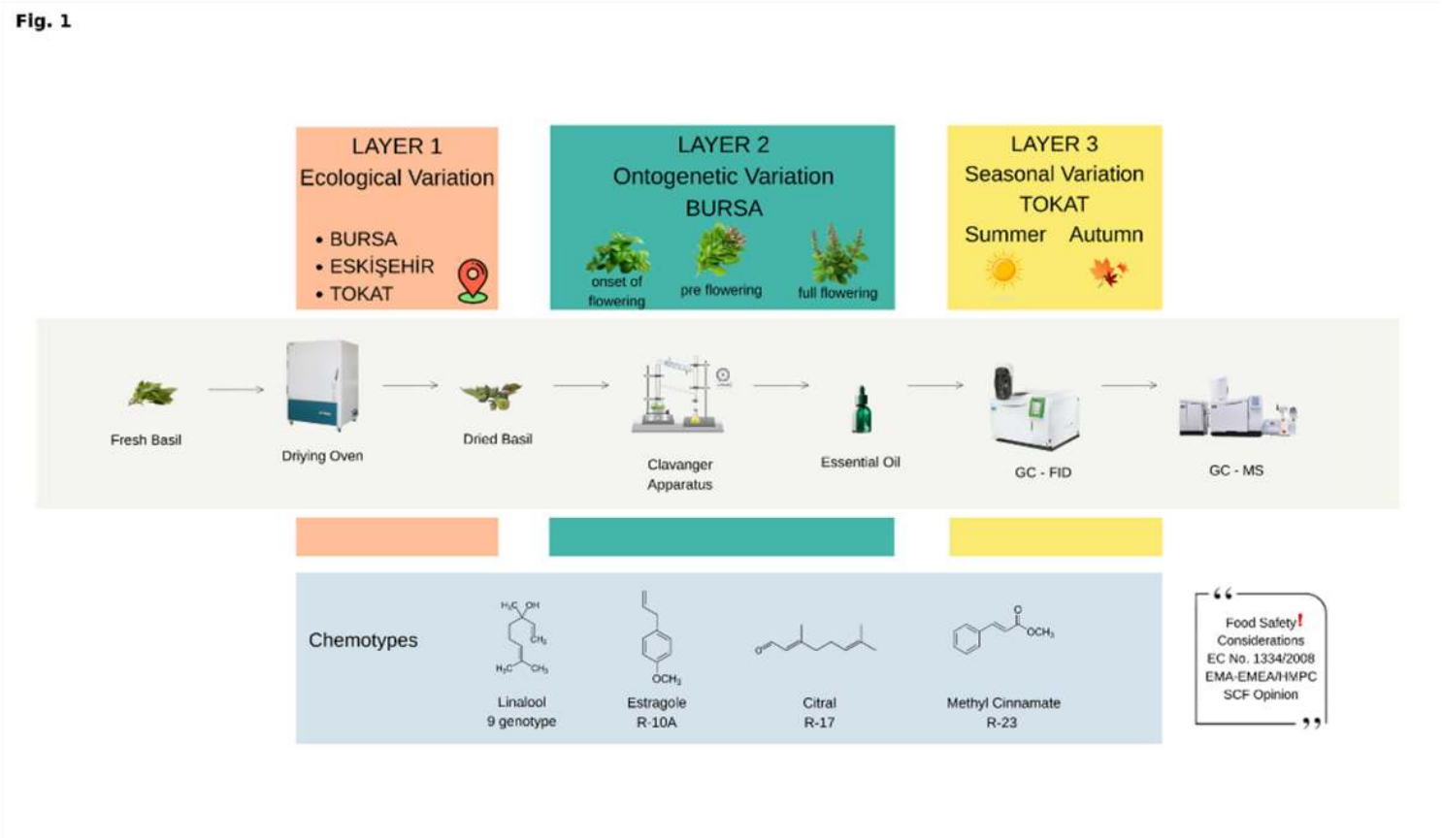


Figure 1

Schematic overview of the three-layer experimental design and analytical workflow used to characterise essential oil yield and chemotype composition in 12 basil (Ocimum basilicum) genotypes. Layer 1 (ecological variation): three locations (Bursa, Eskişehir, Tokat) × two years; Layer 2 (ontogenetic variation): three developmental stages (pre-flowering, onset of flowering, full flowering) at Bursa × two years; Layer 3 (seasonal variation): two harvest seasons (summer, autumn) at Tokat × two years. Fresh plant material was dried, hydrodistilled (Clevenger apparatus), and analysed by GC-FID (quantification) and GC-MS (identification). Four chemotypes were identified: linalool (nine genotypes), estragole (R-10A), citral (R-17), and methyl cinnamate (R-23). Food safety implications are discussed in the context of EU Regulation (EC) No 1334/2008

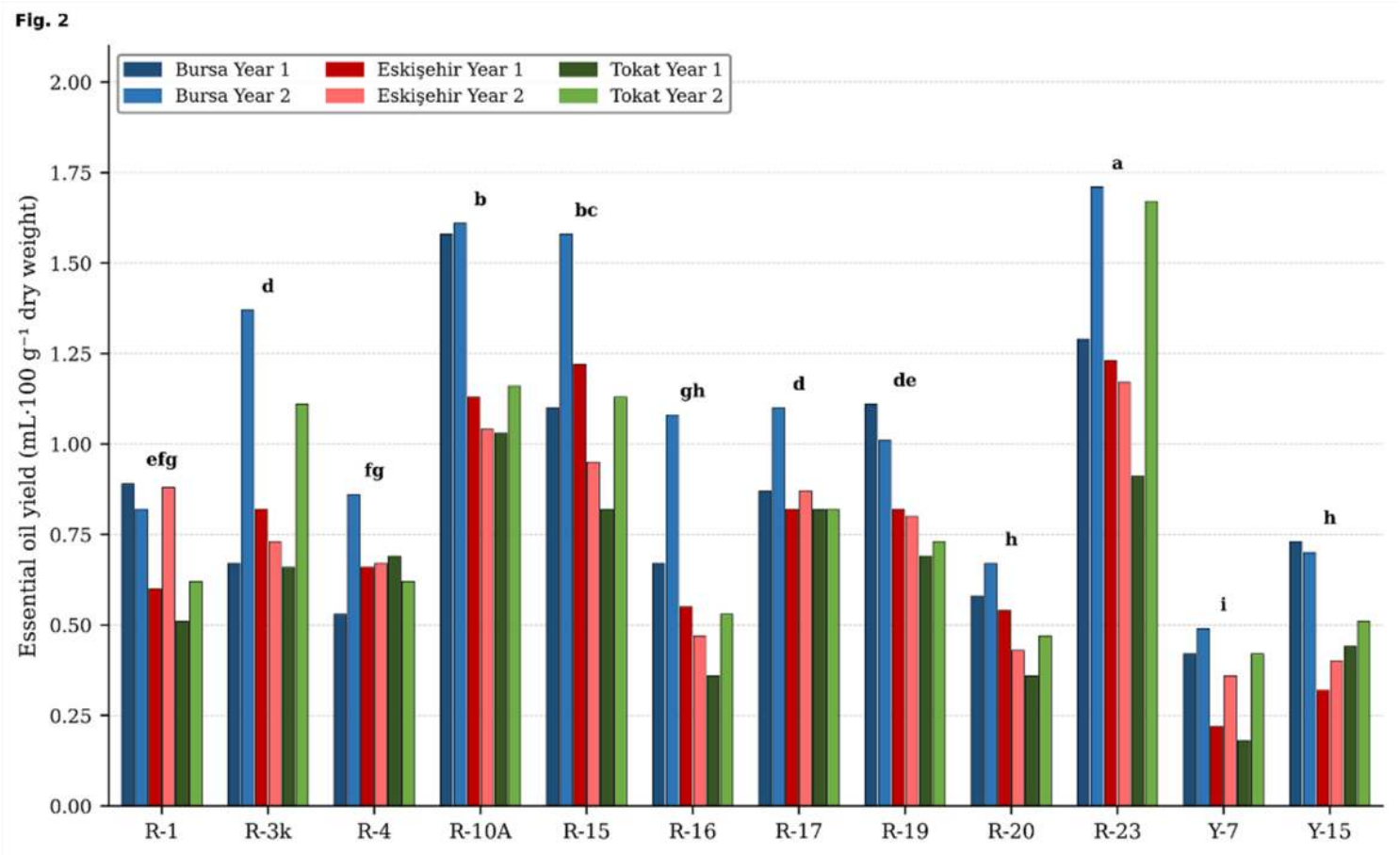


Figure 2

Essential oil (EO) yield (mL·100 g⁻¹ dry weight (DW)) of 12 basil genotypes across three locations (genotype × location interaction; Layer 1 – ecological variation). Bars represent individual year values for each location (Bursa Year 1, Bursa Year 2, Eskişehir Year 1, Eskişehir Year 2, Tokat Year 1, Tokat Year 2). Different lowercase letters above each genotype group denote significant differences among genotype means across all six environment–year combinations (Duncan's multiple range test, $P \leq 0.01$)

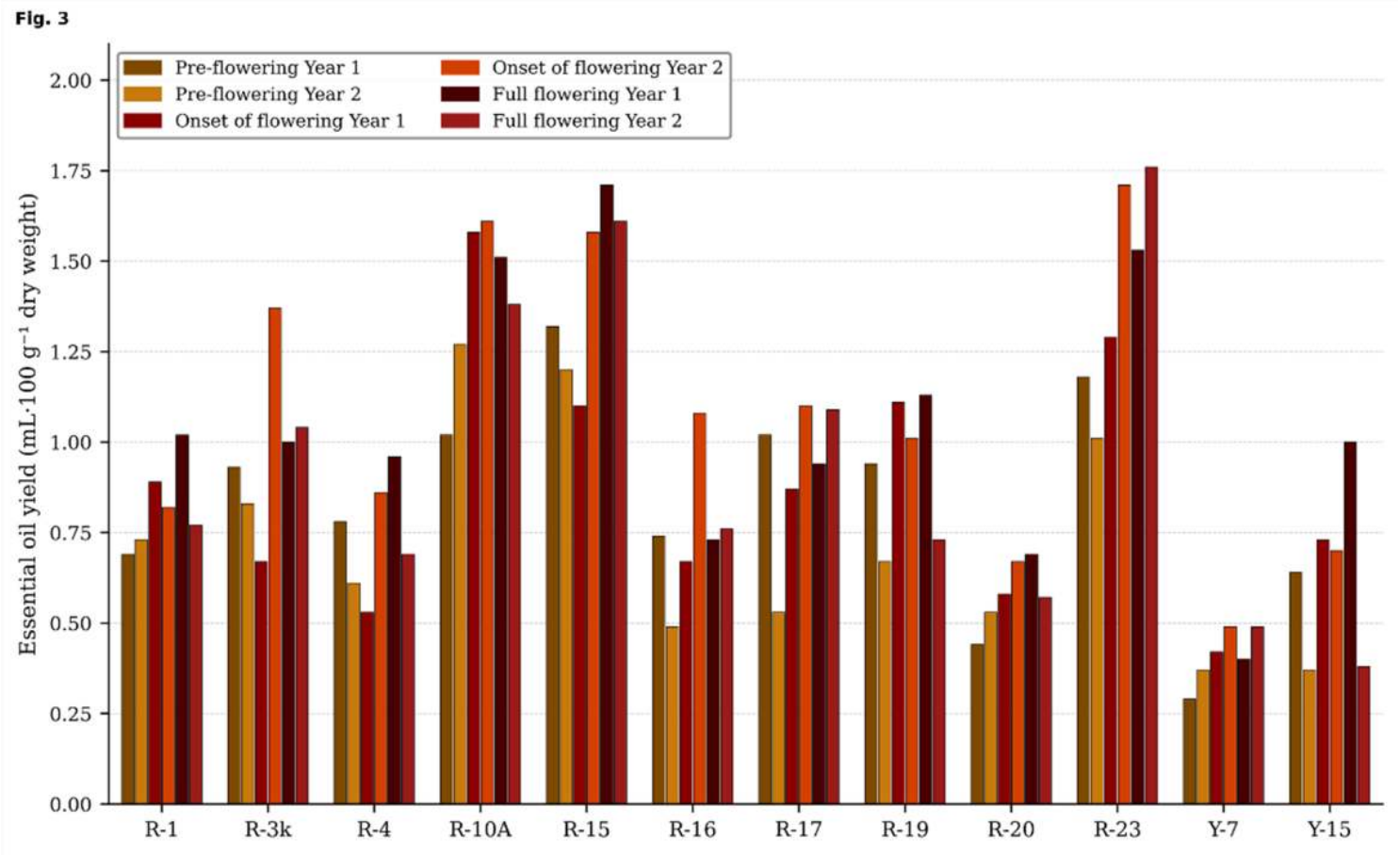


Figure 3

Essential oil (EO) yield (mL·100 g⁻¹ dry weight (DW)) of 12 basil genotypes across three developmental stages at Bursa (genotype × stage interaction; Layer 2 – ontogenetic variation). PF pre-flowering (vegetative), OF onset of flowering (10–15% open flowers), FF full flowering (>50% open flowers), Year 1 first growing year, Year 2 second growing year. Different lowercase letters above genotype groups denote significant differences among stage means (Duncan's test, $P \leq 0.01$)

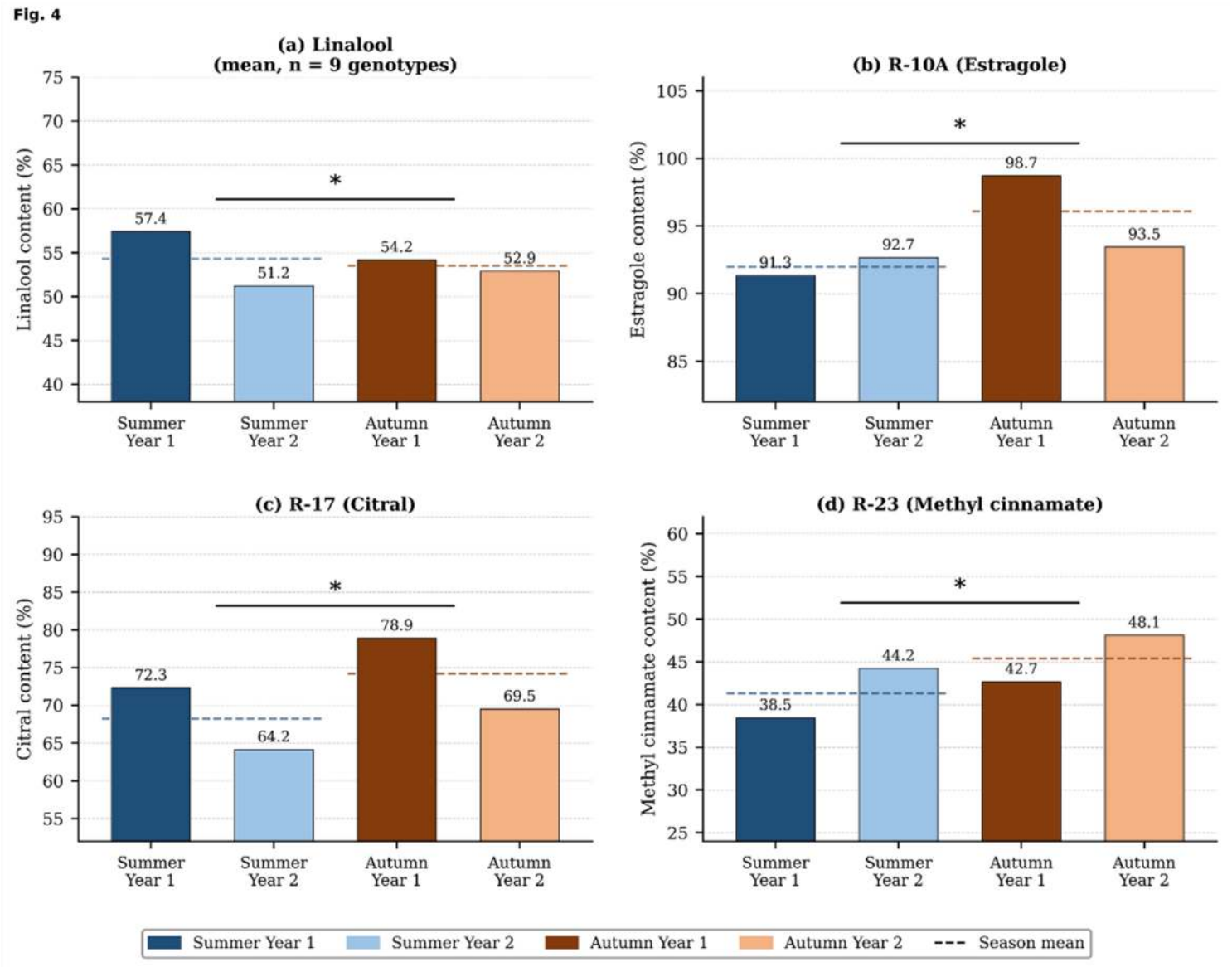


Figure 4

Seasonal variation in the dominant compound relative proportion (%) for each basil chemotype at Tokat (Layer 3 – seasonal variation). a Linalool: mean of nine linalool-type genotypes (n = 9); b Genotype R-10A: estragole; c Genotype R-17: citral; d Genotype R-23: methyl cinnamate. Summer first cut (June–July), Autumn second cut (August–September), Year 1 first growing year, Year 2 second growing year. Individual Year 1 and Year 2 values are shown separately for each season (n = 2 years per season). The brackets with ns denote non-significant seasonal differences (P > 0.05) based on the season main effect in the year × season ANOVA. Dashed lines indicate the seasonal mean for each compound

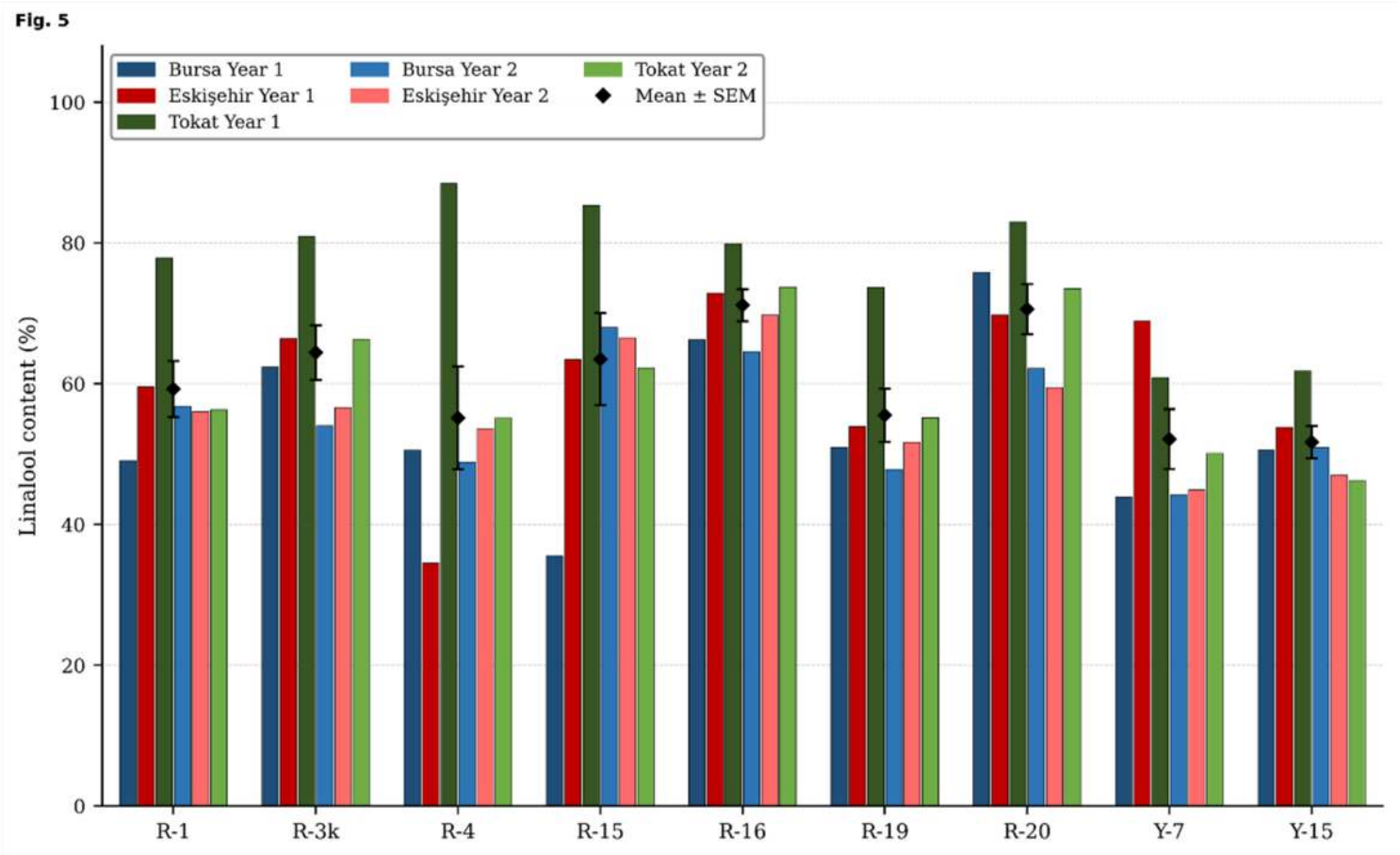


Figure 5

Linalool content (%) of nine linalool-type basil genotypes across three locations and two years (Layer 1 – ecological variation). Six bars per genotype represent Bursa Year 1, Eskişehir Year 1, Tokat Year 1, Bursa Year 2, Eskişehir Year 2, and Tokat Year 2. Error bars show the mean ± standard deviation (SD) across six environment–year combinations (diamond markers)

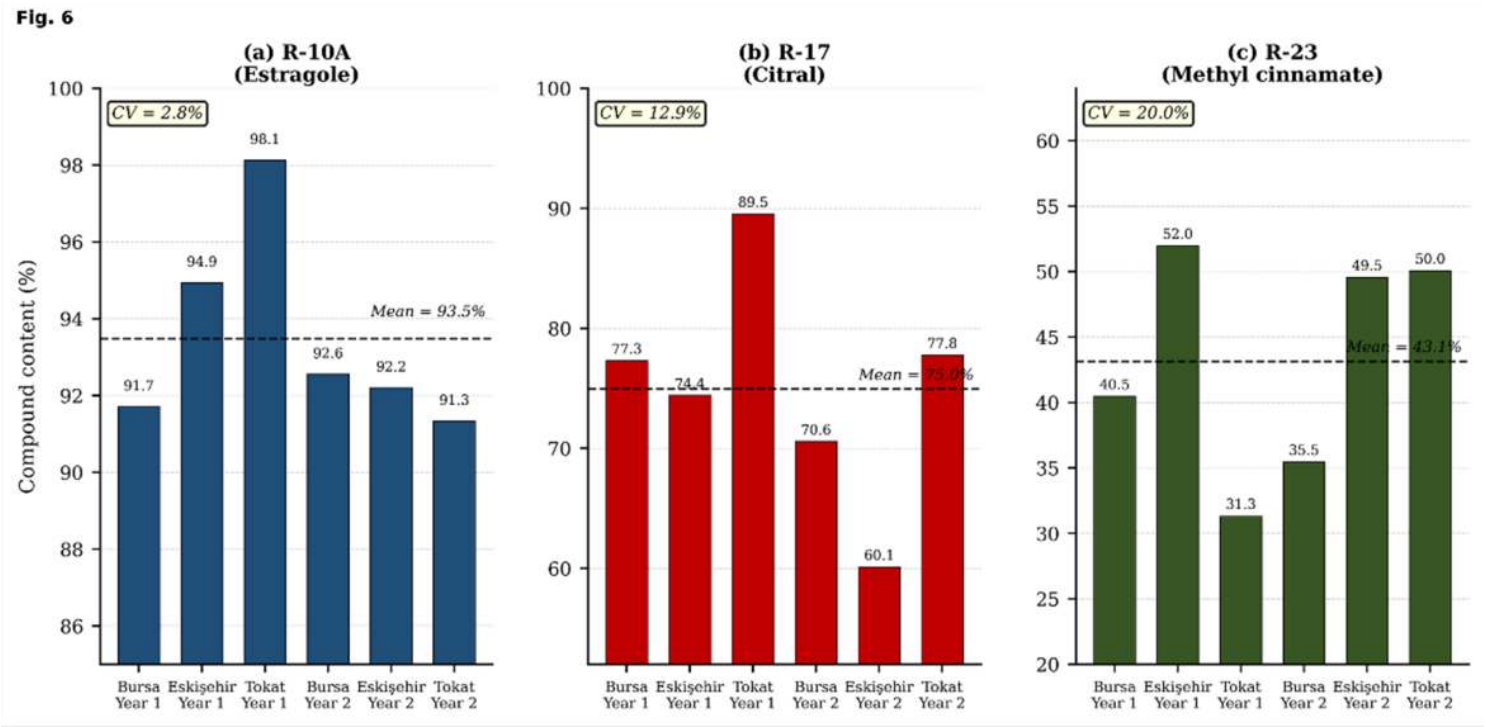


Figure 6

Dominant compound variation in non-linalool chemotypes across three locations and two years (Layer 1 – ecological variation). a Estragole (%) in genotype R-10A (Layer 1 CV = 2.8%); b citral (geranial + neral) (%) in genotype R-17 (Layer 1 CV = 12.9%); c methyl cinnamate (%) in genotype R-23 (Layer 1 CV = 20.0%). Six bars per panel represent Bursa Year 1, Eskişehir Year 1, Tokat Year 1, Bursa Year 2, Eskişehir Year 2, and Tokat Year 2. Dashed lines indicate the overall mean for each chemotype. CV coefficient of variation across six environment–year combinations

Supplementary Files

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- OnlineResource11.pdf